Population structure, host cell interactions and pathogenesis of *Staphylococcus aureus* strains isolated at Tygerberg hospital, South Africa

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DECLARATION

By submitting this dissertation electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extend explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

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SUMMARY

Numerous studies conducted internationally have identified and described several endemic methicillin-susceptible *Staphylococcus aureus* (MSSA) clones. However, only some of these clones are associated with methicillin resistance (CC5, CC8, CC22, CC30 and CC45). To date, studies reporting on the population structure of *S. aureus* isolated in South Africa represent limited demographic areas, focus on methicillin-resistant *S. aureus* (MRSA) only and have displayed little emphasis on virulence. This study was undertaken to elucidate the population structure of *S. aureus* isolated from specific clinical sources at Tygerberg hospital, and to investigate specific host-pathogen interactions of representative isolates.

Consecutive non-repetitive clinical *S. aureus* isolates were collected over one year (September 2009/2010) with patient demographics and limited clinical information. Strains were typed by PFGE and molecular markers (*spa*, multi-locus sequence typing (MLST), *agr*, Staphylococcal Chromosome Cassette *mec* and Panton-Valentine leukocidin (PVL)). Representative isolates were selected and investigated for the presence of virulence genes, adherence (to immobilised fibronectin [Fn], fibrinogen [Fg], collagens IV [CnIV] and VI [CnVI]), cellular invasion and cell death induction. Statistical association were determined between all *in vitro* results and methicillin-resistance, clonality, patient HIV status and bacterial PVL status.

Fifteen percent of the isolates (n = 367) were MRSA. Forty four present of isolates were PVL+. *agr* I-IV and SCC*mec* I-V were identified. The MSSA population was diverse: ST22 (dominant), ST1865 and ST121 were PVL+. ST45, ST1863 and ST15 were PVL-. PVL- MRSA were diverse: ST612-MRSA-IV (dominant), ST5-MRSA-I, ST239-MRSA-III, ST36-MRSA-II and ST22-MRSA-IV. The genes *fnb*A/B (fibronectin-binding protein A/B), *clf*A/B (clumping factor A/B), *eap* (extracellular adherence protein), *nuc* (nuclease), *coa* (coagulase) and *hld* (delta toxin) were detected in all representative isolates.

The CC8 and CC6 isolates adhered strongly to all ligands (100-700% of control, ligand dependent), while isolates of CC45, CC22 and CC88 adhered strongly only to Fg and Fn. The CC30, CC15, and CC12 isolates adhered extremely strongly to CnIV (>300%) and CC8, CC15, and C6 to CnVI (>200%). Isolates from CC30, CC8, CC15, CC6, CC12, CC97, CC88 and CC45 were highly invasive (>100%). ST121 was non-invasive (>50%). Isolates of CC5, CC30 and CC121 were non-cytotoxic (<50%), while isolates of CC22, CC8, CC15, CC45 and CC88 were very cytotoxic (>70%).

No significant difference was observed in adherence or cell death induction of MRSA vs. MSSA clones or between isolates from HIV+ vs. HIV- persons. PVL- isolates displayed higher cellular invasiveness than PVL+ isolates.

The presence of ST612-MRSA-IV, ST22-MRSA-V and ST8-MRSA-V points to local SCC*mec* acquisition, as we found MSSA isolates with the same *spa* types. Numerous MSSA clones were prevalent, but do not appear to have a major common genetic background with MRSA. PVL was highly prevalent among MSSA, indicating acquisition of PVL genes independently of SCC*mec*. The abilities to adhere to specific immobilised ligands *in vitro* were diverse and grouped with the genetic background, while the vast majority of isolates were invasive and induced significant cell death.

We can conclude that the population of *S. aureus* at Tygerberg hospital is composed of a vast number of MSSA and MRSA clones, which display varying patters of adherence to selected ligands and of which, the majority clones are invasive and cytotoxic.

OPSOMMING

Talle internasionale studies het verskeie endemiese metisillien vatbare *Staphylococcus aureus* (MSSA) klone geïdentifiseer en beskryf. Slegs 'n paar van hierdie klone word geassosieer met metisillien weerstandigheid (Klonale kompleks (KK) 5, KK8, KK22, KK30 en KK45). Studies oor die bevolking struktuur van *S. aureus* geïsoleer in Suid-Afrika is tot dusver beperk tot demografiese gebiede, fokus slegs op metisillien-weerstande *S. aureus* (MRSA) en het min klem op virulensie geplaas. Hierdie studie is onderneem om die bevolking struktuur van *S. aureus*, geïsoleer vanaf spesifieke kliniese bronne, in die pasiëntpopulasie van Tygerberg-hospitaal te ondersoek en om ondersoek in te stel na spesifieke gasheer-patogeen interaksies van verteenwoordigende isolate.

Opeenvolgende, nie-herhalende en suiwer kliniese *S. aureus* isolate is versamel oor ´n periode van een jaar (September 2009/2010), tesame met pasiënt demografiese- en beperkte kliniese inligting. Stamme is deur PFGE en molekulêre merkers (*spa*, MLST, *agr*, SCC*mec* en PVL) beskryf. Verteenwoordigende isolate is gekies en ondersoek vir die teenwoordigheid van virulensie gene, aanhegting (aan geïmmobiliseerde fibronektien [Fn], fibrinogeen [Fg], kollageen IV [CnIV] en kollageen VI [CnVI]), sellulêre indringing en die induksie van seldood. Statistiese assosiasies is bepaal tussen alle *in vitro* resultate en methicillin-weerstandigheid, klonaliteit, pasiënt MIV status en bakteriese PVL status.

Fyftien persent van die isolate (n = 367) was MRSA. Vier-en-veertig van die isolate was PVL+. *agr*I-IV en SCC*mec* I-V is geïdentifiseer. Die MSSA bevolking was divers: ST22 (dominant), ST1865 en ST121 PVL +. ST45, ST1863 en ST15 was PVL+. PVL- MRSA was divers: ST612-MRSA-IV (dominant), ST5-MRSA-I, ST239-MRSA-III, ST36-MRSA-II en ST22-MRSA-IV. Die gene *fnb*A/B (fibronektien A/B), *clf*A/B (klontings faktor A/B), *eap* (ekstrasellulêre aanhegtings protein), *nuc* (nukease), *coa* (koagulase) en *hld* (delta toksien) was aangetref in alle verteenwoordigende isolate.

Isolate van KK8 en KK6 het sterk aan alle ligande (100-700% van kontrole, ligand-afhanklike) aangeheg, terwyl isolate van KK45, KK22 en KK88 slegs sterk aand fibronektien en fibrinogeen aangeheg het. Isolate van KK30, KK15, en KK12 het baie sterk aan CnIV (> 300%) aangeheg en KK8, KK15, en KK6 and CnVI (> 200%). Isolate van KK30, KK8, KK15, KK6, KK12, KK97, KK88 en KK45 was hoogs indringend (> 100%). ST121 was nie-indringende (> 50%). Isolate van

KK5, KK30 en KK121 was nie-sitotoksiese (<50%), terwyl isolate van KK22, KK8, KK15, KK45 en KK88 baie sitotoksies was (> 70%).

Geen betekenisvolle verskil is waargeneem in die aanhegting of seldood induksie van MRSA teenoor MSSA klone of tussen isolate van MIV+ teenoor MIV- persone nie. PVL- isolate het hoër sellulêre indringing as PVL+ isolate vertoon.

Die teenwoordigheid van ST612-MRSA-IV, ST22-MRSA-V en ST8-MRSA-V verwys na die plaaslike verwerwing van SCC*mec*, aangesien ons MSSA isolate beskryf het met dieselfde *spa*-tipes. Talle MSSA klone was algemeen, maar het nie 'n beduidende genetiese agtergrond met MRSA vertoon nie. PVL was baie algemeen onder MSSA isolate en die PVL gene is dalk onafhanklik van SCC*mec* verkry.

Die vermoë om aan spesifieke geïmmobiliseer ligande *in vitro* aan te heg was divers en groepeer met die genetiese agtergrond, terwyl die meerderheid van die isolate indringend was en kon betekenisvolle sel dood veroorsaak.

Ons kan aflei dat die bevolking van *S. aureus* by die Tygerberg hospitaal saamgestel is uit 'n groot aantal van MSSA en MRSA klone, wat verskillende patrone van aanhegting aan geselekteerde ligande vertoon en waarvan die meeste klone indringende en sitotoksies is.

RESEARCH OUTPUTS

ORAL PRESENTATIONS:

The following oral presentations were presented (reverse chronological order):

1. **W. Oosthuysen**. "*In vitro* characterisation after population structure analyses of clinical *S. aureus* isolated at Tygerberg hospital, South Africa". 15th International Symposium on Staphylococci and Staphylococcal Infections (ISSSI), 26-30th August 2012, Lyon, France.
2. **W. Oosthuysen**. "*In vitro* characterisation after population structure analyses of clinical *S. aureus* isolated at Tygerberg hospital, South Africa". Stellenbosch University, Faculty of Medicine and Health Sciences Academic Year Day: Pathology session, 14-15th August, 2012.
3. **W. Oosthuysen**. "*In vitro* characterisation after population structure analyses of clinical *S. aureus* isolated at Tygerberg hospital, South Africa". 4th IRTG International Symposium, 24-26th June 2012, Würzburg, Germany.
4. **W. Oosthuysen**. "Host cell invasion and cell death induction by *Staphylococcus aureus"*. MedVet-Staph meeting, 1-2nd December 2011, Neustadt am Rübenberge, Mariensee, Germany.
5. **W. Oosthuysen.** "Molecular Epidemiology of *S. aureus* at Tygerberg Hospital, Western Cape, South Africa". 3rd IRTG1522 International Symposium, 24-26th February 2011, STIAS, Stellenbosch, South Africa.
6. **W. Oosthuysen.** "Population structure, host cell interactions and pathogenesis of *Staphylococcus aureus* strains isolated at Tygerberg hospital, South Africa". 2nd IRTG1522 International Symposium, 14-15th May 2010, Bad Staffelstein, Germany.

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The following poster was presented:

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AWARD RECEIVED:

The following award was bestowed upon Mr. Wilhelm Oosthuysen:

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PLANNED RESEARCH PUBLICATION:

The following manuscript originating from this research have been prepared for publication:

1. Wilhelm F. Oosthuysen, Heidi Orth, Carl J. Lombard, Bhanu Sinha and Elizabeth Wasserman. 2013. Population structure analyses of *S. aureus* at Tygerberg Hospital, South Africa, reveals a diverse population, high prevalence of Panton-Valentine leukocidin genes and the local emergence of MRSA clones. Submitted to the journal Clinical Microbiology and Infection.

2. Wilhelm F. Oosthuysen, Heidi Orth, Carl Lombard, Bhanu Sinha and Elizabeth Wasserman. *In vitro* characterisation of representative clinical South African *Staphylococcus aureus* isolates from various clonal lineages. Submitted to the journal PLoS One.

Mr. Oosthuysen was co-author in the following publications originating from other research projects:

1. K. Karayem, **W.F. Oosthuysen**, K. Hoek, H. Orth and E. Wasserman. 2013. The correlation of blood and nasal *Staphylococcus aureus* isolates in patients admitted to an academic hospital in South Africa. Submitted to the Journal of Clinical Microbiology.
2. H. Orth, Z. Salaam-Dreyer, E. Makgotlho, **W. Oosthuysen**, B. Sinha and E. Wasserman. 2013. Characterization of *Staphylococcus aureus* bacteraemia at Tygerberg hospital. South African Journal of Epidemiology and Infection, vol. 28(1).

DEDICATION

To my family

“Imagination is more important than knowledge.” – Albert Einstein

“Change is the only constant in the world.” - Anonymous

“Once we accept our limits, we go beyond them.” – Albert Einstein

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LIST OF DEFINITIONS

Clonal complex: A group of known strains, such as *S. aureus*, which share a common ancestry, i.e. evolved from the same ancestral cell, as determined by a specific molecular typing method, such as macro-restriction analyses or multi-locus sequence typing.

Intravascular device: An intravascular catheter inserted directly into a vein or artery.

Isolate: A micro-organism obtained in pure culture from a clinical specimen.

Population structure: Clones of *S. aureus* identified in circulation, as determined by specific molecular biology typing techniques (PFGE, *spa* typing, MLST), and the association of these clones with specific clinical categories (such as adult/paediatric clones or clones associated with a specific gender) or specific bacterial characteristics (such as resistance to methicillin).

Prosthetic device: An artificial object inserted into any human body part other than a vein or artery e.g. cardiac pacemaker.

Strain: A group of micro-organisms within a species e.g. *Staphylococcus aureus* which have the same particular characteristic.

LIST OF ABBREVIATIONS

+	positive
-	negative
aa	amino acid
AIDS	Acquired immunodeficiency syndrome
AIP	Auto-inducing peptide
ATCC	American Type Culture Collection
BHI	Brain-heart infusion
BJ	Bone-and-joint
BURP	Based-Upon Repeat Pattern
°C	degrees Celsius
CA	Cardiac
CA-MRSA	Community-associated methicillin-resistant *Staphylococcus aureus*
***cap*5/8**	Capsule 5/8 gene
CBSI	Catheter-related blood stream infection
CC	Clonal complex
CDC	Centres for Disease Control and Prevention
CHIPS	Chemotaxis inhibitory protein
chp	Chemotaxis inhibitory protein gene
***clf*A/B**	Clumping factor A/B gene
cna	Collagen binding protein
CnIV	Collagen type IV

CnVI	Collagen type VI
coa	Coagulase gene
CO_2	Carbon dioxide
CPT	Cape Town
dlv	double-locus variant
DNA	Deoxyribonucleic acid
DMSO	Dimethyl sulfoxide
eap	Extracellular adherence protein gene
Eap	Extracellular adherence protein
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EMRSA	Epidemic methicillin-resistant *Staphylococcus aureus*
ENT	Ear, nose and throat
ESRF	End-stage renal failure
eta	Exfoliative toxin A gene
etb	Exfoliative toxin B gene
EtBr	Ethidium bromide
EY	Eye
Ex	Excluded
FCS	Foetal calf serum
Fg	Fibrinogen
FITC	Fluorescein isothiocyanate
Fn	Fibronectin

***fnb*A/B**	Fibronectin binding pretein A/B gene
Fnbp(s)	Fibronectin-binding protein(s)
h	hour
HA-MRSA	Hospital-associated methicillin-resistant *Staphylococcus aureus*
HCl	Hydrochloric acid
HIV	Human immunodeficiency virus
hla	Alpha toxin gene
hlb	Beta toxin gene
hld	Deta toxin gene
hlg	Gamma toxin gene
***hlg*v**	Gamma variant toxin gene
HREC	Human Research Ethics Committee
HSA	Human serum albumin
hVISA	heterogenous vancomycin-intermediately resistant *S. aureus*
HVR	Hyper-variable region
***ica*A**	Biofilm A gene
ICAM-1	Endothelial cell adhesion molecule
IEC	Immune evasion cluster
IgG	Immunoglobulin-G
incl.	including
IVD	Intra-vascular device
IWG-SCC	International Working Group on the classification of Staphylococcal Cassette Chromosome Elements
kb	kilobase

KZN	KwaZulu Natal
LA-MRSA	Livestock-associated methicillin-resistant *Staphylococcus aureus*
LDH	Lactate dehydrogenase
M	Molar
mg	milligram
mg/ml	milligram per millilitre
$MgCl_2$	Magnesium chloride
MHB	Mueller-Hinton broth
min	minute
ml	millilitre
MLST	Multi-locus sequence typing
mm	millimetre
mM	millimolar
MOI	Multiplicity of infection
MRA	Macro-restriction analyses
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSSA	Methicillin-susceptible *Staphylococcus aureus*
MSCRAMM	Microbial surface components recognising adhesive matrix molecule
µg	microgram
µg/ml	microgram per millilitre
µl	microliter
µM	micromolar
Na	Not applicable
NaCl	Sodium chloride

NCTC	National Collection of Type Cultures
NHLS	National Health Laboratory Service
nm	nanometre
NP	Necrotising pneumonia
ns	not significant
NSBI	Nosocomial blood stream infection
NT	non-typeable
nuc	Nuclease gene
OD	Optical density
o/n	overnight
ORIF	Open-reduction and internal fixation
PBP	Penicillin-binding protein
PBS	Phosphate-buffered saline
PCP	Pneumocystis pneumonia
PCR	Polymerase chain reaction
PEARLS	Pan-European Antimicrobial Resistance Using Local Surveillance
PFGE	Pulsed-field gel electrophoresis
PI	Propidium iodide
PMNs	Polymorphonuclear cells
pmol	picomol
PROST	Prosthetic device
PSM	Phenol-soluble modulins
PTA	Pretoria
PVL	Panton-Valentine leukocidin

rEap	Recombinant extracellular adherence protein
RE	Restriction enzyme
RO	Reverse osmosis
rpm	revolutions per minute
RT	room temperature
s	second
sak	Staphylokinase gene
SCC*mec*	Staphylococcal Cassette Chromosome *mec*
SCIN	Staphylococcal complement inhibitor
scn	Staphylococcal complement inhibitor gene
SCV	Small colony variant
SD	standard deviation
***sdr*C/D/E**	SDR-repeat proteins C/D/E genes
SE	Staphylococcal enterotoxin
sea	Staphylococcal enterotoxin A gene
seb	Staphylococcal enterotoxin B gene
sec	Staphylococcal enterotoxin C gene
sed	Staphylococcal enterotoxin D gene
see	Staphylococcal enterotoxin E gene
seg	Staphylococcal enterotoxin G gene
seh	Staphylococcal enterotoxin H gene
sei	Staphylococcal enterotoxin I gene
sej	Staphylococcal enterotoxin J gene
sel	Staphylococcal enterotoxin L gene

sem	Staphylococcal enterotoxin M gene
SEM	Standard error of the mean
sen	Staphylococcal enterotoxin N gene
seo	Staphylococcal enterotoxin O gene
sep	Staphylococcal enterotoxin P gene
SERAM	Secreted expanded repertoire adhesive molecules
SLST	Single locus sequence typing
***spa*-CC(s)**	*spa*-Clonal complex(es)
SSSS	Staphylococcal Scalded Skin Syndrome
SSTI	Skin and soft tissue infection
ST	Sequence type
TAE	Tris-acetic acid-EDTA buffer
TBE	Tris-boric acid-EDTA buffer
TE	Tris-EDTA buffer
T_m	annealing temperature
UK	United Kingdom
UPGMA	Unweighted pair group method with arithmetic mean
USA	United States of America
UT	Urinary tract
V	Volts
V/cm	Volts per centimetre
VNTR	Variable-number tandem repeat
WT	wild-type
w/v	weight per volume

y	year

CHAPTER 1: General introduction

1.1 The Organism

Staphylococci are Gram-positive cocci, occurring mainly as irregular grape-like clusters, but can also be found singly, in pairs, tetrads or short chains. The organism is non-motile, non-spore forming and as a rule, catalase positive[1]. On solid agar, *Staphylococcus aureus* colonies are usually smooth, slightly raised and often pigmented, ranging from creamy-yellow to orange. *S. aureus* is a facultative pathogenic bacterium that is considered to be one of the most important and significant human pathogens. Although *S. aureus* has successfully adapted as a human pathogen or a coloniser, the organism can quite easily play both roles, i.e. be both a pathogen and coloniser[2, 3]. Humans are natural reservoirs of *S. aureus*, as are cattle and pigs[4]. The most common site of colonisation is the nose, but other sites, such as the nasopharynx, axillae and groin may also be colonised[1]. According to previous studies, carriage of the organism in the anterior nares of up to 50% of adults can be found, of which 30% are persistently colonised[2, 5] by the organism. Van Belkum *et al.*[5] classified human nasal carriage into persistent carriage or colonisation and non-carriers or intermittent carriers.

S. aureus possesses many surface factors, as well as secreted molecules and enzymes which aid with host colonisation and cellular invasion[2, 6] and can eventually lead to a wide range of clinical infections and even death. Infections caused by this organism may involve skin and soft tissue, bone and joints or organs, such as the lungs or kidneys. However, this can easily lead to sepsis, as well as septic shock, which, in turn, is associated with vascular damage and multiple organ failure[7]. Previous staphylococcal infection is not always required, as seen in cases involving food poisoning and toxic shock syndrome[2, 8].

Whole genome sequencing of two related methicillin-resistant *Staphylococcus aureus* (MRSA) isolates revealed that the organism is much more complex in pathogenicity than originally thought. The genome complexity revealed the ability of *S. aureus* to acquire useful genes through lateral gene transfer[9]. Several genomes from clinical and laboratory *S. aureus* isolates have been

sequenced and include methicillin susceptible *Staphylococcus aureus* (MSSA) and MRSA isolates (http://www.ncbi.nlm.nih.gov/genome/154). Basic genome analyses revealed the presence of two domains in the *S. aureus* chromosome: the 1st domain contained the vast majority of house-keeping genes, while the 2nd contained the vast majority of acquired genes responsible for virulence or drug-resistance[10].

After investigating the clonal nature of *S. aureus* using revised multi-locus sequence typing (MLST) data, Feil *et al.* identified no significant difference in the distribution of MLST sequence types (STs) between colonising and invasive isolates[11]. They were also able to conclude that point mutations, and not homologous recombination, are the main mechanism of evolution which gives rise to new alleles, and that homologous recombination plays a role in the long-term evolution of the organism[11].

1.1.1 Methicillin-resistant *S. aureus* (MRSA):

S. aureus infections were initially treated with sulphonamides, which were soon replaced by penicillin. Although initially very effective, resistance through the production of penicillinase was acquired shortly after penicillin's introduction[12]. Resistance to these two drugs lead to the chemical alteration of the penicillin-nucleus, and cloxacillin and methicillin were synthesised, both penicillinase resistant. Yet, once again, resistance was soon acquired and MRSA was born, resistant to most currently licenced classical beta-lactam antibiotics [13].

MRSA emerged as a major clinical and epidemiological hospital-associated problem in the 1980s[2], even though Jevons described the first MRSA isolate in the 1960s[14]. After the identification of *S. aureus* strains resistant to methicillin in Britain in the early 1960s[14], MRSA has become a major global health concern and is commonly associated with serious infections, such as sepsis, endocarditis and osteomyelitis[15]. It was realised during the 1990s, using molecular fingerprinting techniques, that epidemic MRSA clones are capable of spreading between the continents and that a clone can replace another epidemic MRSA clone[16]. Using fingerprinting techniques, scientists in New York City were able to identify MRSA clusters and outbreaks in various hospitals in the city[17]. It was also soon realised that many of these early MRSA clones were widespread across the

planet[18]. This is still the case today. A MRSA clone may be dominant for a while in a given geographic area, where after it is replaced by another MRSA clone. Two different MRSA clones can also co-dominate a specific setting over a period of time[19].

Unlike its counterpart, MRSA possesses the *mecA* gene that confers resistance to nearly all β-lactam antibiotics. The *mecA* gene encodes for an alternative penicillin-binding protein (PBP)-2, namely PBP-2' or PBP-2a, which has a remarkably low binding affinity to β-lactam antibiotics[20]. PBPs are involved in cross-linking of the peptidoglycan of the bacterial cell wall and normally display very high binding capacities to β-lactam antibiotics[20]. The *mecA* gene is located on a mobile genetic element called the Staphylococcal Chromosome Cassette *mec* (SCC*mec*) and was only described in the year 2000[21]. This mobile element's ability to integrate into and excise from the *S. aureus* genome is regulated by the *ccr* cassette (also carried on the element) which encodes for recombinase genes[21]. This makes MRSA quite different from other forms of antibiotic resistance, the fact that the resistance gene (*mecA*) is integrated into the host genome.

The development of a multi-locus sequence typing (MLST) scheme for the typing of *S. aureus* showed that the vast majority of MRSA isolates were part of clonal complex (CC) 5, CC8, CC22, CC30 and CC45[22, 23]. MRSA isolates have also been identified in other CCs, especially community-associated MRSA (CA-MRSA) isolates, which display more diversity in origin. Some clones occur very infrequently and usually in very small numbers, and are regarded as sporadic clones, such as CC509 and ST1048 isolates[23]. No single common genetic factor distinguishes epidemic and sporadic clones[24]. It has also been established that MRSA infections can be seasonal, depending on the geographic region[25].

1.1.1.1 Staphylococcal Chromosome Cassette *mec* (SCC*mec*):

Being able to assign a SCC*mec* type correctly is an important requirement for epidemiological studies. SCC*mec* typing can also be utilised for the investigation into the origin and evolution of contemporary MRSA clones[26].

SCC*mec* was first described as a novel mobile genomic element, capable of site- and orientation-specific integration into and precise excision from any *S. aureus* genome due to recombinase genes of the invertase/resolvase family, called chromosome cassette recombinase genes (*ccrAB* or *ccrC*)[21, 27, 28]. A defining feature of any SCC*mec* type is the presence of two gene complexes, the *mec* complex (responsible for conferring resistance to methicillin through the *mecA* gene) and the *ccr* gene complex (responsible for integration into and excision from the bacterial genome). At least eleven different SCC*mec* types have been identified and described to date. This is based on the combination of the *mec* and *ccr* gene complexes, of which numerous classes and allotypes have been described. Several PCR-based typing schemes have been designed for the identification of SCC*mec* elements in clinical *S. aureus* isolates[29-33] and also for the sub-typing of some SCC*mec* types[34]. Refer to Figure 1.1 (A & B) for a structural comparison of SCC*mec* types I - VIII. Guidelines have been published for the classification of newly discovered SCC*mec* types[35].

SCC*mec* types I, II and III are today still predominantly associated with hospital-associated MRSA (HA-MRSA)[36]. They are the largest of the SCC*mec* elements and carry numerous other genes on them, such as Tn*554* (conferring resistance to erythromycin and spectinomycin), IS*431* (chromosomal deposit site for other resistance genes), and integrated plasmids, such as pUB110 and pT181[37, 38]. SCC*mec* type IV is smaller and commonly devoid of most other resistance genes and was originally associated with community-associated MRSA strains (CA-MRSA)[36, 39]. The description of SCC*mec* type V, also associated with CA-MRSA, lead to the discovery of the *ccrC* gene[27]. This type is also small, lacks additional resistance genes and carries genes for a restriction-modification system[27].

SCC*mec* type VI was originally misclassified as a variant of type IV, but this element possessing a different *ccrAB* allotype lead to its reclassification[40]. SCC*mec* type VII was detected in Japanese

CA-MRSA isolates, was relatively large and found to contain an additional *ccrC* gene[41]. IS*431* and Tn*554* were also identified to be present on this CA-MRSA associated SCC*mec* type[41].

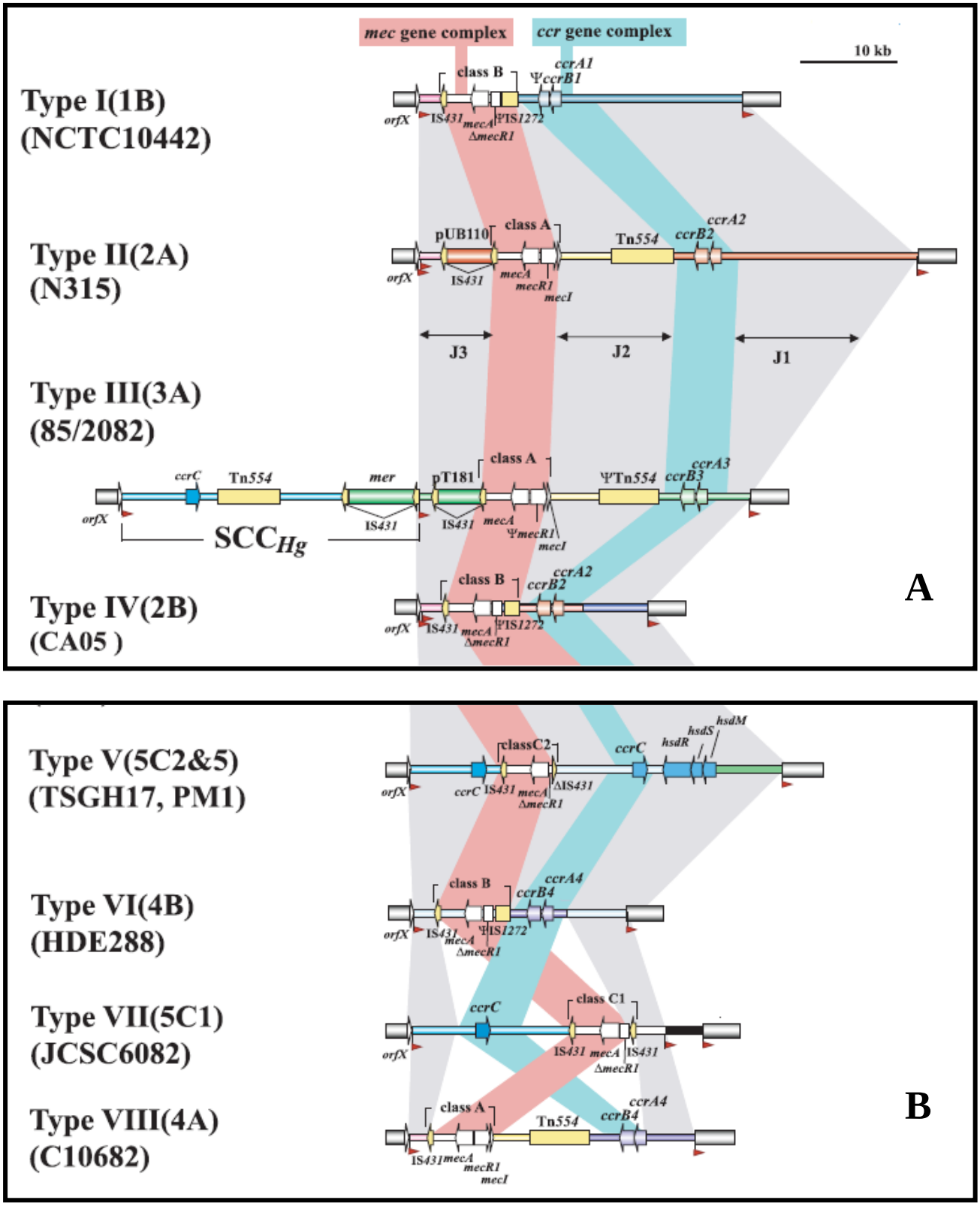


Figure 1.1 Structures of SCC*mec* types I – VI (A) and SCC*mec* types V – VIII (B). Adapted from IWG-SCC[35].

SCC*mec* type VIII was identified in a Canadian epidemic MRSA isolate. Complete sequence data of this element revealed it was generated through recombination[42].

SCC*mec* types IX and X were more recently identified in 2011 in *S. aureus* strains of MLST CC398, commonly associated with livestock-associated MRSA (LA-MRSA) strains[43]. These SCC*mec* types also carried genes conferring resistance to heavy metals and were distinct from those normally found in human *S. aureus* strains[43], suggesting they originated in non-human staphylococcal species. SCC*mec* type XI was discovered in clinical MRSA isolates from the UK and Denmark, as well as bovine milk samples from the UK[44]. This SCC*mec* type carried a *mecA* homologue, designated $mecA_{LGA251}$, arsenic resistance genes and a β-lactamase gene, *blaZ*[44]. ST398 MSSA isolates have also recently been identified in humans, which were not acquired from any animals[45].

Although SCC*mec* is predominantly associated with antibiotic resistance, there are reports of SCC*mec* II and III elements carrying phenol-soluble modulin (PSM) genes[46] (*psm-mec*/PSM-*mec*), which are normally encoded in the core genome of *S. aureus*. *psm-mec* genes have recently also been identified in SCC*mec* II[47] and VIII elements[48]. PSMs are thought to be involved in phagosomal escape, but their exact functions still remain unknown[49]. Otto *et al.* has identified PSMs to be involved in virulence using *in vivo* infection models[49-51].

1.1.1.2 The evolution of MRSA:

Initially it was thought that *mecA* was acquired only once by *S. aureus*[52], but today we know that *mecA* was transferred to various clones of *S. aureus*. Analyses of >3000 *S. aureus* isolates from Latin America, the USA and Europe showed that the Iberian and the Brazilian/Hungarian clones are related to the Archaic clone, the first MRSA clones[53] (ST250-MRSA-I)[54]. The fact that the Archaic (ST250-MRSA-I) and Iberian (ST247-MRSA-I) clones possessed the same SCC*mec* type, SCC*mec* I, while the Brazilian/Hungarian (ST239-MRSA-III) clones possessed SCC*mec* III was more proof that *mecA* was acquired more than once by *S. aureus*[53]. DNA microarray data generated by Fitzgerald *et al.* identified five distinct *S. aureus* clonal backgrounds that have received the SCC*mec* element (i.e. the *mec* gene cluster), thus supporting the theory of multiple independent acquisition events[55]. Analyses of MSSA strains collected during the 1960s revealed

that all early MRSA isolates had the same phenotypic and genetic properties as these MSSA isolates, confirming that the early MRSA isolates were in fact the progeny of these MSSA isolates[56].

MLST analyses of the five lineages identified by Fitzgerald *et al.*[55] indicate that they have evolved from two distinct ancestral lineages, of which the 1st lineages could be traced back to MSSA and MRSA isolates from the UK and Denmark from the 1950s – 1960s[12]. The 2nd lineage had a completely different MLST profile and was identified in MRSA isolates from Japan and the USA (ST5), as well as from paediatric isolates from all over the globe[12]. Four SCC*mec* elements were also identified in unique combinations[12]. Since the identification of the 1st MRSA isolates, numerous MRSA clones have spawned from epidemic MSSA isolates[57]. Some of these MRSA isolates have now acquired resistance against vancomycin, which has for a long time been regarded as the antibiotic of last resort[58].

Robinson *et al.* were able to construct evolutionary models of the emergence of the five dominant MRSA clones, namely CC5, CC8, CC22, CC30 and CC45, by constructing phylogenetic trees of the diversity present in seven *S. aureus* surface proteins (*sas*). They also constructed trees based on MLST data and found that the two trees constructed conveyed the same picture[59]. Using these models they were able to identify at least 20 MRSA emergence events and concluded that the MRSA clones are four times more like to emerge due to SCC*mec* acquisition by sensitive isolates than the replacement of a SCC*mec* element by another[59]. They also identified SCC*mec* IV as the most common SCC*mec* type. In ST5 alone, it has been shown that different SCC*mec* types have been acquired at least on 23 occasions[60]. MLST has become the gold-standard technique to employ for investigating the evolutionary history and origin of MRSA[13, 61, 62].

Although *S. aureus* evolved predominantly through point mutation, two lineages have been described to have evolved though a single chromosomal replacement event of 224 kb and 557 kb respectively[54, 63]. One of these replacement events involved two pandemic MRSA clones, ST30 (CC30) and ST8 (CC8). A 557 kb chromosomal region of ST8, which included the *spa* gene, was replaced by the same region of ST30, leading to the emergence of ST239 (CC8)[63]. It is due to this replacement that ST30 and related STs (*spa* t012) and ST239 (*spa* t037) are clustered in the same

spa-CC. A similar exchange of chromosomal fragments has been reported between CC5 and CC8[64].

1.1.1.3 Community-associated MRSA (CA-MRSA):

Since the early 1990s, CA-MRSA has emerged as a serious community health problem, causing an array of severe invasive infections, particularly those associated with the skin and soft tissue[65] and different forms of necrotising disease, such as necrotising pneumonia[66, 67]. CA-MRSA isolates are usually characterised by the presence of either SCC*mec* IV or V and are usually non-multidrug resistant. Some countries, like the USA, carry a high burden of CA-MRSA infections caused by isolates of the USA300 (ST8) complex[67, 68], which has spread also to other parts of the world[69, 70]. It has recently become clear that the increased virulence seen in isolates of the USA300 and USA500 complexes, when compared to other contemporary MRSA clones, is due to the differential expression of core genome-encoded virulence determinants, such as α-toxin and PSMs, and not primarily due to the acquisition of mobile genetic elements[71], even though PSM has been identified on SCC*mec* elements[48] and PVL is encoded by acquired phages[65, 72]. This has also been confirmed using an *in vivo* rabbit skin abscess model[50, 51].

There is a lack of data regarding CA-MRSA from Africa. CA-MRSA used to be identified based mainly on clinical definitions, proposed by the CDC in 2000. However, recent studies have shown that clinical data alone is not enough to identify CA-MRSA, and that this should be supplemented by molecular typing data, preferentially sequence-based methods such as single-locus sequence typing (SLST) (e.g. *spa* typing), MLST, presence or absence of PVL and type of SCC*mec* carried[73].

It is known that the worldwide CA-MRSA epidemic caused by isolates of the USA300 clone is being driven by MSSA isolates with the same genotypic and phenotypic characteristics[74]. ST8 PVL+ MSSA isolates can very easily become CA-MRSA clones upon the acquisition of a SCC*mec* IV or V element. This is the case for other ST associated with CA-MRSA in Europe and Australia, such as ST80 and ST59[75, 76].

1.1.2 The global regulator *agr*:

Agr is responsible for the regulation of gene expression of numerous I, cytotoxins and enzymes[77]. *agr*-dependant factors are also responsible for the mediation of induction of apoptosis by *S. aureus*[78]. The expression of cell-wall associated adherence proteins are upregulated during the early stages of infection, and once infection has been established, the expression of invasive factors are favoured.

The *agr* system consists of a 3kb locus which contains opposing transcription elements and is driven by two promoters, P2 and P3[77, 79]. The polycistronic P2 transcript is coded from four genes, *agr*A, C, D and B which encodes for a two-component system as well as its autoinducing peptide (AIP). AgrB is a transmembrane protein and is responsible for the processing of the pro-AIP, encoded for by *agr*D, and secretion of the processed AIP. The AIP then binds to the signal receptor AgrC, which leads to the phosphorylation or dephosphorylation of AgrA, the response regulator, which in turn, activates the P2 (RNAII) and P3 (RNAIII) promoters[77, 79]. This quorum-sensing pathway activates transcription of the P3 promoter at high cell density. This is usually a reaction to the environmental concentrations of the secreted AIP. Target cell regulation is achieved by the transcript of the P3 promoter, a RNAIII molecule, which is upregulated by activated AgrA. Activation of RNAIII can result in the increased expression of secreted proteins and a reduced expression of cell surface proteins[80].

1.1.3 Pathogenicity and virulence of *S. aureus*:

S. aureus as a species possesses an exceptionally diverse set of virulence factors, some of which show a variable prevalence. The pathogenesis of infection is influenced by both secreted and structural products alike, as well as the appropriate regulation of their expression. A virulence factor may be involved in several roles during pathogenesis (pleiotropy), or the same virulence function might be performed by several pathogenicity determinants (redundancy)[3]. It is thought that some clones are more virulent than other, although this is controversial, and essentially any *S. aureus* genotype associated with humans can cause life-threatening infection[81].

S. aureus has several surface anchored proteins, named "microbial surface components recognising adhesive matrix molecules" or MSCRAMMs. These molecules, such as clumping factors, collagen and fibronectin-binding proteins, mediate adherence to host tissues[82] to establish an infection. These proteins can interact with an array of host tissue structures, such as collagens, fibronectin, fibrinogen serum- and plasma proteins and many more. MSCRAMMs play a key role during the initiation of endovascular, bone and joint and prosthetic-device infections[3]. Different MSCRAMMs may be able to adhere to the same host-tissue component. Opsonisation may be prohibited by another MSCRAMM, staphylococcal protein A (spA), known for binding the F_c region of eukaryotic IgG[3]. Although it has been shown that spA can be involved in adherence, the exact function of this protein, till today, remains unknown. Polysaccharides associated with biofilms, such as those encoded by the *ica* locus, allow the organism to persist. Growth as a biofilm also aids the organism to evade the host immune system and most antimicrobials[2]. Biofilm-forming strains are mostly associated with prosthetic devices, such as intravascular catheters or pin tracts.

As infection progresses, local tissue destruction, bacterial dissemination and metastatic infection may ensue. The latter two occur, usually as a result of bacterial spread into the bloodstream and penetration into surrounding tissue. Although the basic mechanism of invasion has been described in molecular detail[83], limited data exist linking this to wild-type (WT) strains and human pathology, apart from *in vivo* endocarditis models[84].

Vast arrays of *S. aureus* proteins also contribute to host immune evasion. One key guard is the assembly of antiphagocytic microcapsules, specifically capsule type 5 and 8. These components are involved with abscess formation, provide protection against phagocytic uptake and enhance microbial virulence[85]. A conjugate vaccine has been developed based on capsular polysaccharide 5 and 8. Staphylococcal complement inhibitor (SCIN) and chemotaxis inhibitory protein of *S. aureus* (CHIPS) are two other proteins involved in host innate immune evasion. Both genes are carried on β-hemolysin-converting bacteriophages (β C-ϕs)[86], which are phages that integrate specifically into the β-hemolysin gene, disrupting it and blocking expression of functional β-toxin. SCIN is a C3 convertase inhibitor while CHIPS modulates bacterial chemokine receptors[86]. Both SCIN and CHIPS protect *S. aureus* from the host innate immune system and are thus very important virulence factors. Two other genes are also sometimes found on the same phages (β C-ϕs) and are also involved in circumvention of the host innate immune system, namely *sak* (Staphylokinase) and

sea (or *sep*) (Staphylococcal enterotoxin A or P). Enterotoxin A, which also acts as a superantigen, has the ability to modulate the functions of chemokine receptors, while Staphylokinase has been shown to have numerous functions[86]. Together, these genes form an immune evasion cluster (IEC) and are highly specific to human strains. To impede neutrophil extravasation and chemotaxis, a large number of proteins are secreted, a prominent member of which is the extracellular adherence protein (Eap)[3]. This is a cell-surface associated protein which belongs to the SERAM (secreted expanded repertoire adhesive molecules) group of molecules, is not covalently anchored, and inhibits wound healing, angiogenesis and inflammation. This protein has also been designated major histocompatibility complex class II analogous protein (Map). It binds rather promiscuously to different monomeric matrix macromolecules, although it discriminatively distinguishes specific extracellular matrix aggregates[87], and also serves as an *S. aureus* invasin[88, 89].

Some strains excrete a leukocyte-specific toxin called Panton-Valentine leukocidin (PVL), and strains carrying the genes for this toxin come from diverse backgrounds[90]. This two component toxin induces pores in the membranes of polymorphonuclear neutrophils (PMNs)[91] and is highly leukotoxic[72]. This toxin has been associated with necrotising lesions of the skin and subcutaneous tissues[65], necrotising pneumonia[66, 92] and other skin and soft tissue infection (SSTIs)[93, 94] caused by CA-MRSA strains. However, it is known that PVL- isolates are just as virulent as their PVL+ counterparts in some *in vivo* infection models[95]. A differential effect on neutrophils by PVL is seen when several species are compared (human, rabbit, murine or simian)[91] and it is still thought that PVL plays an important cytotoxic role in CA-MRSA pathogenesis, especially for isolates from the USA300 clone[68, 69]. PVL can be regarded as a good marker of epidemicity, but its causal role in pathogenicity is less clear, i.e. this toxin can be associated with certain genetic backgrounds, but PVL+ strains are not necessarily more virulent than their PVL- counterparts[95, 96]. The *lukS/F*-PV genes are generally well conserved among different genetic lineages[97]. Recent data has shown that PVL+ isolates can be imported, especially from the Tropics and Subtropics, through nasal colonisation and SSTIs[98].

Another toxin with leukotoxic properties is γ-toxin. It has been demonstrated that this toxin causes the direct lyses of erythrocytes and leukocytes[3, 72] and can also be involved in abscess formation and other invasive skin infections[3]. δ-toxin can also lyse erythrocytes[8] and is related to the PSMs[49, 99]. The exfoliative toxins EtA (*eta* being phage-borne) and EtB (*etb* being plasmid-based) are commonly associated as the cause of Staphylococcal Scalded Skin Syndrome (SSSS), usually a superficial skin disorder involving local blistering of the skin[8].

1.1.3.1 Pathogenesis of infection:

In order for *S. aureus* to initiate an infection it first has to find an appropriate site, where it will have to adhere to host tissue. Adherence and colonisation can be mediated by an array of bacterial molecules[100] expressed during the logarithmic growth phase. Novel adhesins are still being described[101]. Adherence, if successful, can result in host cellular invasion by utilising the host cellular integrin $\alpha_5\beta_1$ (alpha5-beta1)[102], which is accessed after the bacterial invasin (*fnbA/B* or *eap*) has bound to fibronectin[103, 104].

The organism can persist outside the cell, for instance during wound infections by growing as a biofilm on host tissue or prosthetic devices[105] which allows for the organism to escape the host immune system, as well as the effect of administered antimicrobials[3]. Once inside the cell, the bacteria can grow and persist, usually in the form of small-colony variants, or SCV, only to revert later to a more virulent phenotype, and kill the cell. *S. aureus* can invade non-professional phagocytes, including epithelial and endothelial cells[102]. The organism can also persist in these cells and cause toxin-mediated diseases, such as toxic shock syndrome, food poisoning or exfoliative disorders.

S. aureus can also kill the cell immediately after invasion. Cell death is usually induced through the expression of various toxins, especially haemolytic toxins (such as α-toxin) and leukocidin (such as PVL)[3]. These toxins are normally expressed during the stationary growth phase.

1.1.3.2 Host cellular invasion:

Although *S. aureus* has classically been regarded as an extracellular pathogen, mainly due to the pathogenesis of infection relying on toxin production, *S. aureus* has in recent years been recognised as a facultative intracellular pathogen[106, 107]. Host cellular invasion can be achieved or hindered by a number of bacterial surface proteins, of which FnbPs, Eap and Pls will be discussed further during Chapter 3. Invasion is usually mediated by bridging of the bacterial protein via fibronectin, to the host cellular receptors, the integrins $\alpha_5\beta_1$[103].

1.1.4 Clinical outcome of *S. aureus* infections:

Clinical outcome of *S. aureus* infection depends not only on host factors, including the site of infection, but also on organism factors, such as virulence factors/traits, regulatory fitness and susceptibility to antibiotics[108]. Apart from having a greater mortality rate, patients suffering from MRSA surgical site infections normally have prolonged period of hospitalisation and also have significantly higher medical expenses[109]. The lack of effective anti-MRSA antimicrobial agents and the complicated nature of MRSA infections may be two contributing factors. Meta-analysis has also shown that patients suffering from bacteremia due to MRSA had a significantly higher mortality rate when compared to their MSSA counterparts[110] and is speculated to be due to the lack of effective treatment options for MRSA infections. After investigating the recurrence of *S. aureus* bacteremia, Chang *et al.* identified valvular heart disease, liver cirrhosis and deep-seated infections as risk factors for relapse[111]. Nafcillin was also identified to be superior to vancomycin for the prevention of persistent bacteremia or relapse of MSSA bacteremia[111]. Persistent *S. aureus* bacteremia is associated with worse clinical outcome, independent of the methicillin susceptibility[112]. *S. aureus* can persist through growth as a biofilm, which has primarily been associated with the *ica* gene cluster[113], . Proteinaceous-based biofilm growth mechanisms have also been described[114]. Biofilms are also common in device-related infections[2].

The following chapter will focus on elucidating the population structure of *S. aureus* isolates from specific clinical sources from the population of patients treated at Tygerberg hospital in the Western Cape Province, followed by the selection of representative isolates, which was investigated for the presence of selected virulence factors. The same representative isolates were used to investigate their various abilities to adhere to immobilised ligands, which is depicted in Chapter 3. Chapter 4 focusses on the cellular invasiveness and host cell death induction abilities of these representative isolates, while correlation analyses between the various *in vitro* results can be found in Chapter 5. Chapter 6 provides general conclusions regarding the completed study.

CHAPTER 2: The population structure of *Staphylococcus aureus* at Tygerberg hospital

2.1 Introduction

2.1.1 *S. aureus* in South Africa:

Very little information is currently available regarding the molecular epidemiology and pathogenicity of *S. aureus* in South Africa. The incidence of methicillin-resistance among a collection of *S. aureus* isolates collected during 1983/84 from seven teaching centres was found to be 15%[115]. This number increased to 39% for strains collected during the autumn of 1996 from different centres[116], and increased again to 41.5% for all isolates tested and 40.4% for blood culture isolates during the SENTRY study in 1998/1999[117]. The PEARLS (Pan-European Antimicrobial Resistance Using Local Surveillance) study indicated methicillin-resistance to be 33.3% for South African strains isolated during 2001/2002[118]. Refer to Table 2.1.

Table 2.1 Summary of previous *S. aureus* studies containing molecular data conducted in South Africa.

Study	Description	Main Finding	Reference
Moodley *et al.* 2010	Molecular characterisation of national MRSA	Various MRSA clones Low PVL among MRSA Low CA-MRSA	[119]
Janse van Rensburg *et al.* 2010	Molecular characterisation of MRSA from CPT	ST612 dominant MRSA	[120]
Makgotlho *et al.* 2009	Genotyping MRSA from academic hospital (PTA)	SCC*mec* II-IV Low PVL/CA-MRSA	[121]
Shittu *et al.* 2009	Molecular characterisation of mupirocin-resistant MRSA (KZN)	Mupirocin-resistance encoding plasmid present in isolates	[122]

Table 2.1 cont. Summary of previous *S. aureus* studies containing molecular data conducted in South Africa.

Shittu *et al.* 2009	Molecular characterisation of MRSA (KZN)	ST239-MRSA-III; ST5-MRSA-III Novel MLST STs (MRSA)	[123]
Shittu *et al.* 2006	Molecular characterisation mannitol-negative MRSA (KZN)	SCC*mec* IV Mannitol-negative *S. aureus*	[124]

MRSA = methicillin-resistant *S. aureus*; PVL = Panton-Valentine leukocidin; CA-MRSA = community-associated methicillin-resistant *S. aureus*; CPT = Cape Town; ST = sequence type; PTA = Pretoria; SCC*mec* = Staphylococcal Cassette Chromosome *mec*; KZN = KwaZulu-Natal; MLST = multi-locus sequence typing

The first documented case of a hVISA (heterogenous vancomycin-intermediately resistant *S. aureus*) clinical infection in South Africa, and also for the African continent, was reported in 2005, where a 67 year old male suffered a relapse after treatment with vancomycin for MRSA ventriculitis[125]. Eighteen MRSA isolates tested positive for the hVISA phenotype, also in 2005, from an academic hospital. These isolates formed part of *S. aureus* isolates collected from significant clinical sites during the beginning months of 2001 from a large South African teaching hospital[126].

A South African study on MRSA isolates collected nationally to investigate clonal dominance and relatedness of these strains, revealed the presence of several internationally described MRSA clones, such as ST5-MRSA-I, ST239-MRSA-III, ST612-MRSA-IV, ST36-MRSA-II and ST22-MRSA-IV[119]. However, no MRSA isolates were included from Tygerberg hospital and this study excluded MSSA isolates.

Twenty years ago the incidence of MRSA at Tygerberg Hospital was 31% among *S. aureus* strains isolated from blood cultures collected during 1985[127]. For 2008, that number was as high as 44% for blood culture isolates and 29% for isolates from all specimens (laboratory statistics, NHLS Microbiology Laboratory, Tygerberg Hospital). A genotypic study of 113 *S. aureus* blood culture isolates at Tygerberg Hospital by *spa* typing identified 49 different *spa* types that grouped into seven clusters[128]. *Spa* type t037, usually associated with the Brazilian epidemic MRSA clone[13], was identified as the dominant *spa* type. ST612 was also identified as a dominant MRSA clone in another study from Cape Town[120] and has previously only been identified in Australia[129] and South Africa[119]. High rates of methicillin-resistance was noted among HIV+ *S. aureus* carriers

with 77.3% of nasopharyngeal *S. aureus* isolates from HIV+ children in Cape Town resistant to cloxacillin[130].

SCC*mec* type II and type III possessing MRSA isolates have previously also been described in Gauteng by Makgotlho *et al.*[121]. Some information is available for MRSA isolates from KwaZulu-Natal (KZN) province. SCC*mec* type IV was identified among mannitol-negative MRSA isolates collected between 2001 and 2003[124] and ST5-MRSA-III and ST239-MRSA-III clones were identified in mannitol-positive isolates collected at the same time[123]. This data correlates with what has been published by Moodley *et al.*[119]. Also, MSSA isolates expressing low and high levels of resistance to mupirocin have been identified in KZN province[122].

2.1.2 *S. aureus* and HIV/AIDS:

It is important to investigate co-infection of *S. aureus* and HIV due to the high prevalence of HIV in South Africa and the Western Cape. A 2007 ante-natal survey (burden of disease, Department of Health, RSA) determined that on average, 28% of the South African population is HIV+, whereas 13% of the Western Cape population is infected with the virus.

Despite the frequency with which *S. aureus* is isolated in the clinical laboratory, there is a lack of published data regarding the association of *S. aureus* and HIV infection, as well as the clinical presentation of *S. aureus* infections in people living with HIV/AIDS. It appears as if HIV+ persons are at an increased risk for *S. aureus* colonisation and infection[131]. A rise in nasal colonisation by *S. aureus* of HIV-positive children in the Western Cape has been reported[130].

Gordon *et al*. described two unique *S. aureus* clones which were capable of persistently colonising patients living with HIV/AIDS in a suburban drug treatment centre[132]. These clones also caused significant disease as they have been associated with outbreaks in the past. Gordon *et al.* also speculated that these patients act as a reservoir for these clones upon their introduction back into the community[132].

When investigating persistent *S. aureus* nasal carriage in HIV+ patients, Melles *et al.* found the population structure of strains from HIV+ persons to be corresponding strongly with isolates from healthy individuals[133]. However, a higher rate of persistent colonisation (as defined by culture rule of two nasal swabs two weeks apart) rate was identified among HIV+ patients when compared to

that of healthy individuals in the same geographic region. One factor independently determining higher risk of persistent carriage was male sex, while current smoking, Pneumocystis pneumonia (PCP) prophylaxis (low-dose intermittent Cotrimoxazole) and antiretroviral therapy were associated with a lower risk of persistent carriage[133]. Numerous MSSA MLST CCs were identified, all Panton-Valentine leukocidin negative (PVL-).

S. aureus has also been identified as the most common organism isolated from HIV-positive patients admitted to 17 Italian infectious diseases wards with nosocomial blood steam infections (NBSIs) and catheter-related blood stream infections (CBSI)[131]. An uncommonly brutal necrotising fasciitis case involving an HIV+ patient was reported by Olsen *et al.*, which was caused by a PVL+ MRSA clone of the USA300/*spa*1 (ST8) genotype, normally associated with CA-MRSA[134]. They found that although this clone is associated with CA-MRSA infections, an increase in severity of the infections was noted in patients living with HIV/AIDS as *S. aureus* is not frequently reported as the cause of necrotising fasciitis. However, the USA300/*spa*1 genotype has previously been reported as the causative agent of necrotising fasciitis in HIV+ patients[135]. This genotype has also been reported as the cause of the largest number of recurrent CA-MRSA infections in a single HIV+ patient, who experienced 24 episodes of soft tissue infections[136] and has also been associated with endocarditis among HIV+ patients with CA-MRSA bacteraemia[137]. The frequency of SSTI caused by CA-MRSA can also be much higher among HIV+ patients when compared to their healthy counterparts and HIV infection has been suggested as a risk factor for CA-MRSA infection[138].

2.1.3 Molecular typing of *S. aureus*:

In order to elucidate the population structure of any infectious agent, it is necessary to investigate the molecular epidemiology of representative strains isolated from the study population, as phenotypic tests cannot adequately discriminate between various strains. To achieve this, molecular typing techniques are required for the effective discrimination and characterisation of isolates in order to establish clonal relatedness and clonal dominance. Numerous techniques have been described for *S. aureus* typing, of which macro-restriction analysis (MRA) with pulsed-field gel electrophoresis (PFGE) is still regarded as the "gold-standard" for it is still the most discriminatory classical technique available for *S. aureus*[139-141] and is fairly inexpensive to perform. Typing

systems are characterised according to the following criteria: typeability; reproducibility; discriminatory power; ease of interpretation; data portability and ease of use [139, 142]. However, it has been shown that no one typing method is clearly superior above all the rest [139]. PFGE has lost support due to the technique being very labour intensive, as well as the requirement for experienced labour and for normalised and curated restriction pattern databases. As a result of this, there is a tendency towards sequence-based methods, e.g. *spa* typing (the most prominent example for single locus sequence typing) or multi-locus sequence typing (MLST) to discriminate between *S. aureus* isolates [140, 141], due to the increasing accessibility and affordability of DNA sequence technology and the ease with which data can be exchanged between laboratories. However, these techniques do not always discriminate between strains as effectively as PFGE.

2.1.3.1 Macro-restriction analysis (MRA) with pulsed-field gel electrophoresis (PFGE):

Using this technique for the typing of *S. aureus* isolates firstly requires the macro-restriction of the bacterial genome by an infrequently cutting restriction enzyme (RE), mostly *Sma*I. The fragments are then separated according to size on an agarose gel using the technique PFGE, which was first applied for the separation of yeast chromosomes[143]. The current running through the gel is applied at an angle of 120°, resulting in the movement of these large DNA fragments through the agarose matrix. The patterns obtained after staining of the gel are then compared and relatedness of strains can be established[142].

Although PFGE is currently still regarded as the most discriminatory technique to use for the typing of *S. aureus*, it is very labour intensive, requires specific expertise as well as normalised databases. No standard international protocols exist, therefore standardised protocols were developed in the USA[68] and Europe[144], where the technique is also used for outbreak investigation in many clinical settings because of its high discriminatory ability. It can also be applied to investigate outbreaks caused by other organisms such as *Pseudomonas* spp. and *Klebsiella* spp.[142].

The use of PFGE, especially for outbreak investigation, is aided by standardised protocols, together with previously published guidelines for the interpretation of PFGE restriction patterns[142], and for computer software settings[145]. The technique can be applied to *S. aureus* originating from any animal, and is commonly used for establishing clonal relatedness of livestock-associated MRSA (LA-MRSA)[4, 146]. The RE *Apa*I is used for isolates which are non-typeable by *Sma*I digestion.

2.1.3.2 *spa* typing:

The *spa* gene encoding for Staphylococcal Protein A, is found in all *S. aureus* strains. The complete sequence of this gene has previously been described[147, 148] and this revealed a 58-amino acid (aa) unit responsible for immunoglobulin G (IgG) binding and a 2-16-aa unit possibly involved with cell wall binding, named the X-region[148]. The X-region was found to consist of two parts, a variable repetitive region (X_r) and a constant region (X_c). *Spa* typing[149] is based on the amplification and sequencing of the polymorphic sequence of the X_r region, which is composed of several 21-24 base pair (bp) repeats bordered by well-conserved regions[150].

This single-locus sequence-based method is rapid, repeatable, easily portable and standardised, and can be applied in both local and global epidemiological studies[151], as the technique is capable of detecting genetic micro- and macrovariation. The technique has also been validated in terms of outbreak investigations. It was found that *spa* typing was able to discriminate molecular and epidemiologically linked strains quickly and easily[152], but requires a curated database (referred to as the "*spa* server").

Computer software has been designed to facilitate the analysis and interpretation of DNA nucleotide sequences[153]. This software also contains a specific algorithm which can be used to establish clonal relatedness from *spa* repeat units[154]. When compared to other typing techniques, *spa* typing was found to be a very powerful technique for the typing of MRSA[155].

A comprehensive overview of *spa* typing, including experimental procedure, can be found in the book chapter of Hallin *et al*[156]. Please refer to Figure 2.1 for a schematic representation of the *spa* gene and the variable-number tandem repeat (VNTR) region of this gene used for determining strain relatedness.

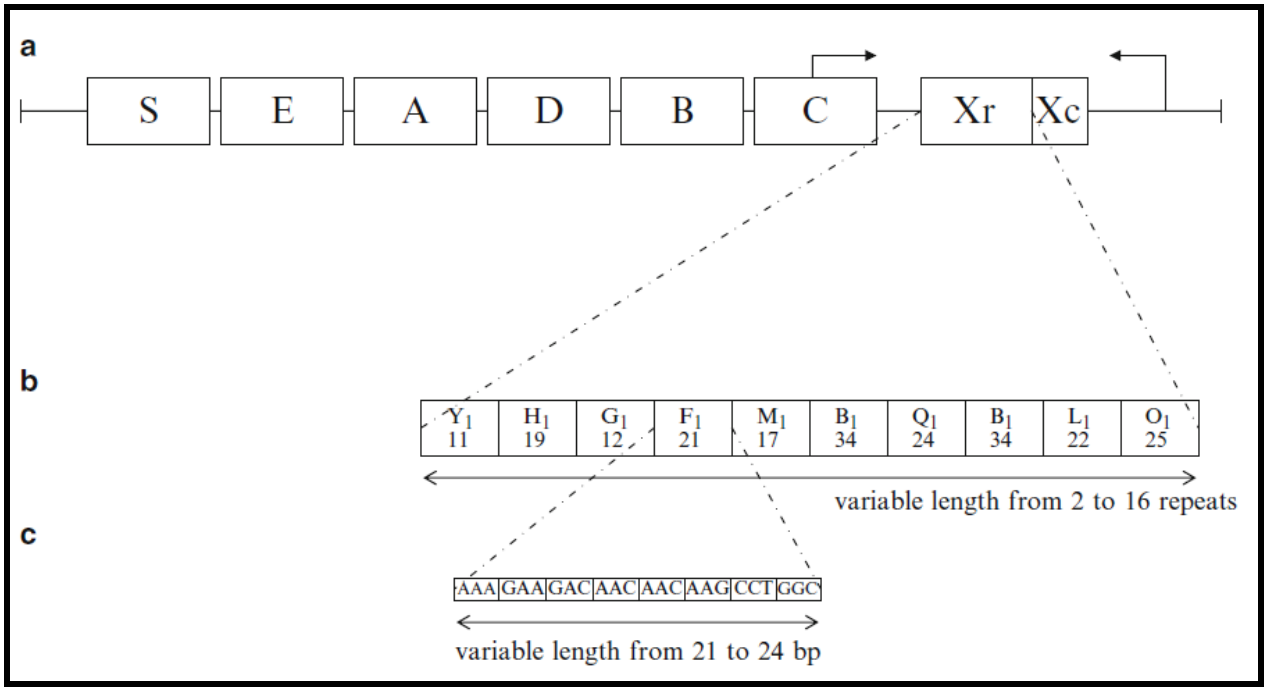


Figure 2.1 A schematic representation of the *spa* gene, adapted from Hallin *et al*[156]. **(a)** The different segments of the *spa* gene: S = signal sequence; A-E = IgG binding domains; X = C-terminal part (X_r is the VNTR region, while X_c is the constant region). The arrows indicate the cross-localisation of primers. **(b)** Repeat structure of the X_r region, with Kreiswirth (YHGFMBQBLO) and Ridom (t008) nomenclature. **(c)** DNA nucleotide sequence of repeat r21/F_1.

2.1.3.3 Multi-locus sequence typing (MLST):

A MLST typing scheme was developed for *S. aureus*[22] in 2000 by Enright *et al*. and is based on the polymorphisms identified in seven housekeeping gene sequences. Clustering of both MSSA and MRSA isolates by MLST agreed well with results obtained by PFGE, confirming that MLST was a typing method with high discriminatory power. Using this technique, Enright *et al*. also established that the population structure of *S. aureus* is essentially clonal in nature and that a few epidemiologically distinct lineages house MRSA isolates[22]. Because of this, MLST was also found to be a good technique to study global epidemiology and evolution of *S. aureus*[22]. Also, new implementations of the algorithm eBURST have allowed scientists to divide a MLST data set of any size into groups of related isolates, called clonal complexes (CC), and to predict the founding or ancestral genotype of each CC[157].

Although a more complicated and expensive technique, the sequencing of seven housekeeping genes provides the advantage that MLST can be used to answer questions regarding the evolutionary biology of bacterial strains. However, its expense and labour intensity prohibits the technique to be applied in outbreak investigations and routine diagnostics[57, 158].

2.1.3.4 *agr* typing:

A hypervariable region is present in the P2 operon, resulting in 4 *agr* variants (I, II, III and IV), and represents a microbial interference mechanism. The AIP from an *agr* type can only activate the *agr* response of strains with the same *agr* type and represses the *agr* response of strains with different *agr* types[80]. This evolutionary divergence occurred early in the development of the species[159]. Persons colonised with *S. aureus* in their anterior nares are usually colonised with strains possessing the same *agr* variant[160]. The *agr* variant of each *S. aureus* strain collected can be determined using a previously published protocol[160].

2.1.4 *S. aureus* in the Western Cape province:

In Cape Town and its surrounding areas, a large proportion of bone and joint (BJ) infections, as well as skin and soft tissue infections (SSTI) appear to be caused by *S. aureus* and are characterised by a complicated course. Patient characteristics associated with persistent infection[112], inappropriate therapy[111] or distinct populations of *S. aureus* strains, including MRSA, may all contribute to complicated infection with *S. aureus*.

Because we currently lack data describing the population structure or molecular epidemiology of *S. aureus* in this setting, and since studies to investigate the pathogenicity or virulence of dominant clones which are part of this population of *S. aureus* have not been performed to date, it is very difficult to correlate patient outcome with a specific *S. aureus* clone or to associate patient or clinical characteristics to clonality. Numerous *spa* types have previously been identified at Tygerberg hospital in a study of blood culture isolates[128]. *Spa* type t037, commonly associated with the Brazilian/Hungarian clone (ST239-SCC*mec* III)[13], was identified as the dominant *spa* type among MRSA isolates[128]. However, during this study only blood cultures isolates were analysed.

2.2 Aims and Objectives

2.2.1 Study aim:

The aim of the research presented in this chapter was to elucidate the population structure of *S. aureus* isolated from specific clinical sites of infection (but excluding blood cultures) in the patients at Tygerberg hospital using a variety of molecular biology techniques and to investigate the potential association between dominant clones and specific bacterial and clinical categories.

2.2.2 Study objectives:

The objectives of this part of the study were the following

1. Collect and store a bank of representative *S. aureus* clinical isolates, together with methicillin-resistance and patient specific information, including HIV status.
2. To perform molecular fingerprinting of these isolates through macro-restriction analyses (MRA) with pulsed-field gel electrophoresis (PFGE) and protein A typing (*spa*) of all isolates collected and multi-locus sequence typing (MLST) of representative isolates.
3. To compare the molecular fingerprints to:
   (a) Determine strain relatedness; and
   (b) Establish clonal dominance.
4. To supplement the molecular fingerprinting data by screening all collected isolates through PCR for:
   (a) The SCC*mec* type present in isolates phenotypically characterised as MRSA;
   (b) The toxin Panton-Valentine leukocidin (PVL); and
   (b) The *agr* variant present in each isolate.
5. To perform statistical analyses and investigate if any correlation exist between clonality and the following categories:
   (a) Methicillin-resistance (MRSA and/or MSSA);
   (b) Clones associated with HIV infection;
   (c) Derived paediatric and/or adult clones;
   (d) Clones associated with a specific sources of infections;
   (a) Clones associated with a specific gender; and

(b) Clones associated with a specific PVL status, i.e. PVL+ and/or PVL- clones.

6. To randomly select a representative isolate and investigate through PCR the presence of numerous virulence determinants from:
   (a) Each major and intermediate PFGE clonal complex;
   (b) Any additional clone(s) associated with any of the categories mentioned in #5 above.

2.3 Materials and Methods

2.3.1 Study design and setting:

This was a cross-sectional study undertaken in the molecular laboratory of the Division of Medical Microbiology of the Faculty of Medicine and Health Sciences, Stellenbosch University, located at Tygerberg Hospital. Tygerberg hospital is a 1200 bed academic hospital in the Western Cape that serves a diverse population.

2.3.2 Ethical considerations:

Ethical approval was granted by the Health Research Ethics Committee (HREC) of the Faculty of Medicine and Health Sciences of Stellenbosch University on the 25th March 2010 (N10/03/066). Approval was granted for the collection of clinical *S. aureus* isolates as well as patient specific information. Each isolate collected was assigned a specific study number in an Excel spread sheet created to ensure that the identity of the patient remained anonymous. Exemption for patient consent was obtained on the following grounds: (1) patient de-lineated samples would be collected; (2) patient information collected for this study was obtained using the National Health Laboratory Service (NHLS) laboratory information system.

2.3.3 Sample collection and patient demographics:

S. aureus isolates were collected from the routine clinical specimens sent to the diagnostic microbiology laboratory of the NHLS at Tygerberg Hospital for diagnostic purposes, from 10 September 2009 until 6 September 2010. Identification and susceptibility testing were done using the VITEK II system (BioMerieux, France). Susceptibility testing was performed using the disc diffusion method. Methicillin susceptibility was interpreted according to CLSI guidelines[161]. Following isolation and identification, collected isolates were sub-cultured on blood agar plates to determine purity. Only pure cultures were stored at -80°C using the MicroBank system (Pro-Lab Diagnostics, Canada). For each isolate methicillin susceptibility was recorded in an Excel spread sheet as well as the following information obtained from the laboratory request form and/or hospital

patient information system: the type of specimen and source of the infection. The following patient demographics were captured: age, gender and HIV status (if available). Only one isolate was included per patient.

2.3.4 Chemicals, reagents and media:

All chemicals and reagents used in this study were of analytical and/or molecular biology grade. The composition of all media is given in section 2.3.17. All media used for bacterial growth was obtained from Greenpoint NHLS Media laboratory (Greenpoint, South Africa). The composition of all buffers is given in section 2.3.17.

2.3.5 Bacterial strains and growth conditions:

The *S. aureus* strains used in this study as controls are listed in Table 2.3. All strains were sub-cultured on 5% Blood agar plates (Greenpoint Media, NHLS, Cape Town) after collection and incubated overnight at 37 ºC. For the extraction of DNA, 10 ml Brain Heart Infusion (BHI) broth (Greenpoint Media, NHLS, Cape Town) was inoculated and incubated overnight at 37 ºC.

2.3.6 Total genomic bacterial DNA extraction:

Total genomic DNA was extracted from each isolate collected by using a previously published, adapted, rapid lysis method[162]. Briefly, 10 ml BHI was inoculated with a single bead from the freezer culture and incubated at 37 °C overnight (o/n). Thereafter, 1 ml of the bacterial broth was transferred to a tube, centrifuged at 14 000 rpm for 2 min and the supernatant aspirated. The pellet was resuspended in 50 µl lysostaphin (100 µg/ml) (Sigma, USA) and incubated at 37 °C. After 30 min, 50 µl Proteinase K (100 µg/ml) (Promega, USA) and 150 µl Proteinase K buffer was added to the bacterial-lysostaphin suspension and incubated again for 30 min at 37 °C. Thereafter, the bacterial suspension was incubated at 100 °C for 10 min, centrifuged at 14 000 rpm for 2 min and the DNA concentration measured using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, USA).

2.3.7 Macro-restriction analyses (MRA) and pulsed-field gel electrophoresis (PFGE):

MRA with pulsed-field gel electrophoresis (PFGE), the "gold-standard" technique used for typing *S. aureus*, was used to obtain a banding pattern after genomic DNA digestion for each isolate. Previously published protocols were used[4, 68, 146]. The banding patterns were used to establish isolate relatedness through using the criteria as published by Tenover *et al*[142]. To aid with gel image analyses and construction of UPGMA (Unweighted Pair Group Method using Arithmetic averages) dendograms, GelCompar II version 6.0 (BioNumerics, Applied Maths, Sint-Martens-Latem, Belgium) was used. MRA with PFGE was performed on all isolates collected.

2.3.7.1 Agarose plug preparation:

10 ml BHI was inoculated with a single bead from the stock culture and incubated at 37°C overnight (o/n). The cell suspension concentration was adjusted to an OD_{610} absorbance reading of 0.9 using a cell density spectrophotometer (Ultrospec 10, GE Healthcare Life Sciences, USA). The adjusted cell suspension was centrifuged at 14 000 rpm for 2 min and the supernatant aspirated. The bacterial pellet was re-suspended in 300 µl 1× TE buffer and equilibrated for 10 min. A 1.8% (wt/vl) agarose (Lonza, Switzerland) solution was made in TE buffer and kept at 55°C in a water bath. 4 µl lysostaphin (Sigma, St. Louis, USA) and 300 µl of the 1.8% agarose was added to the equilibrated cell suspension, gently mixed, and dispensed into the wells of a small, disposable mold. Two plugs were made per isolate. After allowing the plugs to solidify at room temperature for more than 15 min, they were removed and placed in a tube containing at least 4 ml EC lysis buffer and incubated at 37 °C for at least 4 h. The EC lysis buffer was replaced by a wash buffer (TE buffer) and agitated on an orbital shaker (MRC Laboratory Equipment, Israel) at 200 rpm for 30 min. This was repeated three more times. The plugs were prepared for restriction digestion or stored at 4 °C.

2.3.7.2 *Sma*I and *Apa*I restriction enzyme digestion:

A 2-3 mm slice was cut off each agarose plug using a sterile scalpel blade and placed in a 1.5 ml tube, covered with 200 µl equilibration buffer [1× Buffer Tango (*Sma*I) or Buffer B

(*Apa*I)(Fermentas Life Sciences, Maryland, USA)] and equilibrated at room temperature for 30 min. The equilibration buffer was removed and replaced with 200 µl restriction enzyme solution (30U *Sma*I in 1× Buffer Tango or 30U *Apa*I in 1× Buffer B) (Fermentas Life Sciences, USA) and incubated according to the manufacturers recommendations for 4 h (*Sma*I @ 25 °C; *Apa*I @ 37 °C). PFGE was performed directly after the enzyme digestion.

2.3.7.3 Pulsed-field gel electrophoresis (PFGE):

A 1% (w/v) pulsed-field certified agarose (Bio-Rad Laboratories, USA) solution was made in 0.5× TBE buffer (diluted from 10× TBE buffer) and equilibrated at 55 °C. The plug slice was loaded directly on the end of the comb tooth (30 tooth comb). At least three size standards (*S. aureus* strain NCTC 8325-4) were included in each gel. After all the plug slices were loaded onto the comb, the comb was placed into the comb holder and the equilibrated agarose poured carefully into the gel casting. The gel was allowed to solidify at room temperature for 1 h, where after it was subjected to PFGE. 2 l 0.5× TBE buffer was poured into the CHEF DR-III apparatus and allowed to cool to 14 °C (approx. 1 h). PFGE was performed using a contour-clamped homogenous electric field apparatus, i.e. a CHEF DR-III system (Bio-Rad Laboratories, USA). The running parameters used were as follows: *Sma*I: 200 V (6 V/cm); initial switch, 5 s; final switch, 40 s; temperature at 14 ºC; run time, 21 h; *Apa*I: 200 V (6 V/cm); initial switch, 5 s; final switch, 15 s; temperature at 14 ºC; run time, 20 h. After electrophoresis, the gel was stained for 30 min in 500 ml reverse osmosis (RO) water containing 50 µl ethidium bromide (EtBr) (10 mg/ml) (Sigma, USA) and de-stained for 30 min in 500 ml RO water only.

2.3.7.4 PFGE gel analysis and UPGMA dendrogram construction:

The gel was visualised in a Bio-Rad Gel Doc (Bio-Rad Laboratories, USA) system using the Quantity One v.4.6.2 (Basic) software package (Bio-Rad Laboratories, USA) and saved as a TIFF image for further analyses using the GelCompar II version 6.0 (BioNumerics, Belgium) software package. The first, middle and last lane[145] of each gel was the reference size standard NCTC 8325-4, which was used to normalise each gel. Percent similarities were identified through the Dice coefficient and a UPGMA dendogram was created to visualise clusters. The band position

tolerance was set at 1.5% and optimisation at 0.5%. The pulsed-field clusters were identified at a similarity coefficient of 80%[68]. Pulsed-field clusters were assigned alphabetical names, THW-A to THW-EE.

2.3.8 *spa* typing:

spa typing involves sequencing of the hyper-variable region (HVR) of the X-region of the *spa* gene, analysis of the DNA sequence through bioinformatics and assigning a specific number to the sequence, thus elucidating the *spa* type[152, 156] after synchronisation of the local database with the *spa* server (www.ridom.de/spa-server/). *spa*-type nomenclature according to Harmsen *et al.* was used[153]. Subsequently, *spa* types were clustered into *spa*-Clonal Complexes (*spa*-CC) by the algorithm BURP (Based Upon Repeat Pattern). *spa* typing was performed on all isolates collected.

2.3.8.1 DNA amplification and detection:

Total genomic DNA was extracted as in section 2.3.6. PCR reactions were set-up in the PCR laboratory of the Division of Medical Microbiology, NHLS Tygerberg Hospital. All PCR reactions were performed on PCR thermal cyclers, either a Verity (Applied Biosystems, USA) or a GeneAmp PCR system 9700 (Applied Biosystems, USA). A total volume of 25 µl was used containing the following components: 12.5 µl GoTaq PCR Master mix (Promega, USA) containing 1.5 mM $MgCl_2$; 1 pmol of each primer; 1 µl crude DNA; and PCR grade water. A negative control containing all reagents but no DNA was included in each run. Primer set 1[149] (Table 2.2) was used to screen all isolates. In instances where no amplification was observed with primer set 1, set 2 or 3[156] (Table 2.2) was used.

The following cycle conditions were used: initial denaturation at 95 °C for 6 min; 30 cycles of denaturation at 95 °C for 45 s, primer annealing at 62 °C for 45 s and extension at 72 °C for 60 s; and a final extension at 72 °C for 6 min. The PCR reactions were kept at 4 °C until they were analysed.

PCR products were analysed by agarose gel electrophoresis and separated on a 1% (wt/vl) agarose gel in 1× TAE (diluted from 50× TAE) at 100 V for 1 h. 5 µl ethidium bromide (10 mg/ml) (Sigma) was added per 100ml of agarose solution. 5 µl of each PCR reaction was subjected to

electrophoresis and a 100 bp molecular weight marker (Fermentas Life Sciences, USA) was included in the 1st lane of each gel. Gel images were visualised with a Bio-Rad Gel Doc (Bio-Rad Laboratories) using the Quantity One v.4.6.2 software package, or with an Alliance 2.7 Imaging system (UVITEC, UK) using the Alliance v.14.15 software package (UVITEC, UK). All gel images were saved as TIFF files.

2.3.8.2 DNA nucleotide sequencing:

The 20 µl of each PCR reaction remaining was used for DNA sequencing. DNA nucleotide sequencing was outsourced to Inqaba Biotechnologies (South Africa), where the PCR fragments were purified and sequenced using the same forward and reverse primers using during the PCR reaction. Chromatograph sequence files received from Inqaba Biotechnologies were visually inspected with the RIDOM StaphType software package before subjected to bioinformatics-based analyses for *spa* type elucidation.

2.3.8.3 DNA nucleotide sequence analysis:

The software package RIDOM StaphType v.2.1.1 (RIDOM GmbH, Germany)[153] was used for *spa* type elucidation and assignment of clonal complexes (CC). After input of the sequence file (ABI format), the software searches for a 5' and 3' signature sequence indicating the beginning and end of the repeat units. Thereafter, the repeat units were identified, the *spa* type assigned and the sequence saved in the local database. *Spa* types were clustered into *spa*-CC using the built-in algorithm BURP (Based Upon Repeat Patter), which aligns the *spa* types and groups them into clusters. *Spa* types were clustered at a cost of less or equal to 5 repeats and *spa* types shorter than four repeats were excluded from analyses, as these types were too short to deduce ancestry.

2.3.9 Multi-locus sequence typing (MLST):

MLST involved the sequencing of internal fragments of seven housekeeping genes and assigning a unique number to each allele identified, thus allowing for the elucidation of a specific sequence type (ST) based on the allelic profile. MLST was performed according to a previously published protocol[22]. Allelic profiles were obtained using the website http://saureus.mlst.net. Subsequently, sequence types (ST) were clustered into clonal complexes (CC) using eBURST v.3., available at http://saureus.mlst.net/eburst. MLST was performed on a randomly selected representative of each major (>10 PFGE types) and intermediate (4 – 9 PFGE types) PFGE CC. MLST was not performed on PFGE clones classified as minor clones. Two isolates were selected from THW-C as two subgroups, based on *spa*-typing) were identified within this PFGE clone.

2.3.9.1 DNA amplification, detection and nucleotide sequencing:

Total genomic DNA was extracted as in section 2.3.6. PCR reaction were set-up and amplicons detected as in section 2.3.8.1. Primer sets 4-10 (Table 2.2) were used at a T_m of 55°C. DNA nucleotide sequencing was performed as described in section 3.8.2.

2.3.9.2 DNA nucleotide sequence analysis:

ST were clustered into a CC if they had 6/7 similar allelic profiles. All ST identified were also compared to all ST available on the MLST server and again, ST were clustered into a CC if they had 6/7 similar allelic profiles.

2.3.10 SCC*mec* typing:

SCC*mec* typing was performed according to a previously published protocol[29] to allow for the rapid and easy identification of the SCC*mec* types I-VI element present in the genome of each MRSA strain collected. SCC*mec* typing was performed on MRSA isolates only. This multiplex

PCR-based protocol employs ten primer sets, of which each set is specific to the *mec* complex, *ccr* complex, J1 region or J3 region of one SCC*mec* type only.

Total genomic DNA was extracted as in section 2.3.6. PCR reactions were set-up and amplicons detected as in section 2.3.8.1. However, 1 mM $MgCl_2$ (Fermentas Life Sciences, USA) was added to the master mix. Primer sets 11-20 (Table 2.2) were used at a T_m of 53°C. PCR products were analysed by agarose gel electrophoresis and separated on a 3% (wt/vl) agarose gel in 1× TAE (diluted from 50× TAE) at 100 V for 3 h. Apart from the negative control and clinical isolates, one positive control for each SCC*mec* type (I-VI) (Table 2.3) was included in each run.

2.3.11 *agr* typing:

The *agr* variant present in each *S. aureus* strain collected was identified using a previously published PCR protocol[160]. This protocol utilises a universal forward primer for all four *agr* types and four reverse primers, one specific for each *agr* type.

Total genomic DNA was extracted as in section 2.3.6. PCR reaction were set-up and amplicons detected as in section 2.3.8.1. Primers 21-25 (Table 2.2) were used at a T_m of 56 °C and a concentration of 5 pmol/µl each primer. PCR product were analysed by agarose gel electrophoresis and separated on a 2% (wt/vl) agarose gel in 1× TAE (diluted from 50× TAE) at 100 V for 2 h. Apart from the negative control and clinical isolates, one positive control for *agr* type I-IV (Table 2.3) was included in each run.

2.3.12 PCR detection of Panton-Valentine leukocidin (PVL):

All isolates collected were screened through PCR for the toxin PVL according to a previously published protocol[65]. Primer set 26 was used and these primers allowed for the simultaneous detection of *lukS*-PV and *lukF*-PV[65].

Total genomic DNA was extracted as in section 2.3.6. PCR reactions were set-up and amplicons detected as in section 2.3.8.1. Primer set 26 (Table 2.2) was used at a T_m of 62 °C. PCR product were analysed by agarose gel electrophoresis and separated on a 1% (wt/vl) agarose gel in 1× TAE

(diluted from 50× TAE) at 100 V for 1 h. Apart from the negative control and clinical isolates, strain MW2 (Table 2.3) was included with each run as a positive control.

2.3.13 PCR detection of selected virulence factors:

S. aureus possess an array of virulence factors, involved in various different functions, including adherence, invasion, tissue destruction and evasion of the host immune system[82, 85, 86, 113, 163-167]. The same representative isolate of each major and intermediate PFGE CC which was randomly selected for the MLST analyses (see section 2.3.9) was also used for the PCR detection of numerous virulence factors.

Total genomic DNA was extracted as in section 2.3.6. PCR reactions were set-up and amplicons detected as in section 3.8.1. Primer sets 27 to 64 (Table 2.2) were used. All virulence factors were screened through uniplex PCR, except for the ones mentioned in the next paragraph. PCR products were analysed by agarose gel electrophoresis and separated on a 1% (wt/vl) agarose gel in 1× TAE (diluted from 50× TAE) at 100 V for 1 h. Apart from the negative control and clinical isolates, a selected isolate was included with each run as a positive control (Table 2.4).

The genes *coa*, *nuc* and *sed* were simultaneously detected through multiplex PCR. 0.5 pmol of primer was used for *coa* and 2 pmol of primer for *nuc* and *sed*. *sea* and *sec* were also simultaneously detected though multiplex PCR. 1 pmol of primer was used for each gene. s*eg*, *sej* and *sel* were detected through multiplex PCR. 1 pmol of primer used for each gene. *hld* and *hlg* was simultaneously detected through multiplex PCR. 0.5 pmol primer was used for *hlg* and 2 pmol primer for *hld*. An additional 1.5 mM $MgCl_2$ (Fermentas Life Sciences, USA) was added to all of the above mentioned multiplex PCR, bringing the final $MgCl_2$ concentration to 3 mM. The PCR products for all multiplex reactions were separated using a 3% (wt/vl) agarose gel at 100 V for 3 h in 1% TAE buffer.

During set-up and optimisation of the PCR assays, a control isolate was selected as a positive control. All PCR amplicons were first subjected to DNA nucleotide sequencing, as in section 2.3.8.2, to verify the identity of the PCR amplicon, before the selected primer pairs were used to determine the presence of the specific virulence factor in the clinical isolates. Please refer to Table 2.4 for the PCR annealing temperatures used for each virulence factor.

2.3.14 Statistical analyses:

2.3.14.1 Associations between clonality and clinical/bacterial characteristics:

The Fisher's exact test was used to investigate if any association existed between clonality and the following clinical/bacterial characteristics:

(a) Methicillin-resistance (MRSA and/or MSSA);
(b) Clones associated with HIV infection;
(c) Derived paediatric and/or adult clones;
(d) Clones associated with a specific sources of infections;
(e) Clones associated with a specific gender; and
(f) Clones associated with a specific PVL status, i.e. PVL+ and/or PVL- clones.

A confidence interval of 95% was used and an association between clonality and a category was regarded as statistically significant if a p-value < 0.05 was obtained. For all statistical investigations, the software package Stat v.12 was used and performed by Prof C. Lombard of the Biostatistics Unit of the Medical Research Council.

2.3.14.2 Associations between virulence factors and methicillin-resistance or clonality:

The Fisher's exact test was also used to investigate if any associations existed between the virulence factors and methicillin-resistance (i.e. MSSA and/or MRSA), and also between the virulence factors and clonality. A confidence interval of 95% was used and an association was regarded as statistically significant if a p-value < 0.05 was obtained.

2.3.14.3 Discriminatory power of typing techniques:

A discriminatory index (DI) with confidence intervals (CI) was calculated for PFGE and *spa* typing as published previously[168].

2.3.15 List of PCR primers used during the study:

Table 2.2 PCR primers used during this study.

#	Primer	Sequence (5'-3')	PCR (bp)	Application	Source
1	1095F	AgACgATCCTTCggTgAgC	200-500	*spa* typing	[153]
	1517R	gCTTTTgCAATgTCATTTACTg			
2	239F	ACTAggTgTAggTATTgCATCTgT	± 1200	*spa* typing	[156]
	1717R	TCCAgCTAATAACgCTgCACCTAA			
3	1084F	ACAACgTAACggCTTCATCC	± 550	*spa* typing	[156]
	1618R	TTAgCATCTgCATggTTTgC			
4	arcC-F	TTgATTCACCAgCgCgTATTgTC	550	MLST	[22]
	arcC-R	AggTATCTgCTTCAATCAgCg			
5	aroE-F	ATCggAAATCCTATTTCACATTC	500	MLST	[22]
	aroE-R	GgTgTTgTATTAATAACgATATC			
6	glpF-F	CTAggAACTgCAATCTTAATCC	550	MLST	[22]
	glpF-R	TggTAAAATCgCATgTCCAATTC			
7	gmk-F	ATCgTTTTATCgggACCATC	500	MLST	[22]
	gmk-R	TCATTAACTACAACgTAATCgTA			
8	pta-F	GTTAAAATCgTATTACCTgAAgg	550	MLST	[22]
	pta-R	GACCCTTTTgTTgAAAAgCTTAA			
9	tpi-F	TCgTTCATTCTgAACgTCgTgAA	500	MLST	[22]
	tpi-R	TTTgCACCTTCTAACAATTgTAC			
10	yqi-F	CAGCATACAggACACCTATTggC	550	MLST	[22]
	yqi-R	CgTTgAggAATCgATACTggAAC			
11	CIF2-F2	TTCgAgTTgCTgATgAAgAAgg	495	SCC*mec* I; J1	[29]
	CIF2-R2	ATTTACCACAAggACTACCAgC			
12	ccrC F2	gTACTCgTTACAATgTTTgg	449	SCC*mec* V; *ccr*	[29]
	ccrC R2	ATAATggCTTCATgCTTACC			
13	RIF5 F10	TTCTTAAgTACACgCTgAATCg	414	SCC*mec* III; J3	[29]
	RIF5 R13	ATggAgATgAATTACAAggg			
14	SCCmec V J1-F	TTCTCCATTCTTgTTCATCC	377	SCC*mec* V; J1	[29]
	SCCmec V J1-R	AgAgACTACTgACTTAAgTgg			

Table 2.2 cont. PCR primers used during this study.

15	dcs F2	CATCCTATgATAgCTTggTC	342	SCC*mec* I, II, IV, VI; J3	[29]
	dcs R1	CTAAATCATAgCCATgACCg			
16	ccrB2 F2	AgTTTCTCAgAATTCgAACg	311	SCCmec II, IV; *ccr*	[29]
	ccrB2 R2	CCgATATAgAAWgggTTAgC			
17	kdp F1	AATCATCTgCCATTggTgATgC	284	SCC*mec* II; J1	[29]
	kdp R1	CgAATgAAgTgAAAgAAAgTgg			
18	SCCmec III J1-F	CATTTgTgAAACACAgTACg	243	SCC*mec* III; J1	[29]
	SCCmec III J1-R	gTTATTgAgACTCCTAAAgC			
19	mecI P2	ATCAAgACTTgCATTCAggC	209	SCC*mec* II, III; *mec*	[29]
	mecI P3	gCggTTTCAATTCACTTgTC			
20	mecA P4	TCCAgATTACAACTTCACCAgg	162	*mec*A control	[29]
	mecA P7	CCACTTCATATCTTgTAACg			
21	agrF	ATgCACATggTgCACATgC	-	*agr* I-IV	[160]
22	agrR1	gTCACAAgTACTATAAgCTgCgAT	439	*agr* I	[160]
23	agrR2	TATTACTAATTgAAAAgTgCCATAgC	572	*agr* II	[160]
24	agrR3	gTAATgTAATAgCTTgTATAATAATACCCAg	321	*agr* III	[160]
25	agrR4	CgATAATgCCgTCCTACCCg	657	*agr* IV	[160]
26	luk-PV-1	ATCATTAggTAAAATgTCTggACATgATCCA	433	PVL +	[65]
	luk-PV-2	gCATCAASTgTATTggATAgCAAAAgC			
27	fnbA-F	ggCCAAAATAgCggTAACC	228	*fnb*A	[163]
	fnbA-R	gTgAATATgTggCACACTg			
28	fnbB-F	ggAgCggCCTCAgTATTCTT	201	*fnb*B	[82]
	fnbB-R	AgTTgATgTCgCgCTgTATg			
29	clfA1	gTAggTACgTTAATCggTT	210	*clf*A	[164]
	clfA2	CTCATCAggTTgTTCAgg			
30	clfB-F	TggCggCAAATTTTACAgTgACAgA	404	*clf*B	[113]
	clfB-R	AgAAATgTTCgCgCCATTTggTTT			
31	eap-CON1	TACTAACgAAgCATCTgCC	200	*eap*	[165]
	eap-CON2	TTAAATCgATATCACTAATACCTC			
32	cna F	TTCACAAgCTTggTATCAAgAgCATgg	452	*cna*	[113]
	cna R	gAgTgCCTTCCCAAACCTTTTgAgC			
33	sdrC F	CgCATggCAgTgAATACTgTTgCAgC	731	*sdr*C	[113]
	sdrC R	gAAgTATCAggggTgAAACTATCCACAAATTg			

Table 2.2 cont. PCR primers used during this study.

34	sdrD F	CCACTggAAATAAAgTTgAAgTTTCAACTgCC	467	*sdr*D	[113]
	sdrD R	CCTgATTTAACTTTgTCATCAACTgTAATTTgTg			
35	sdrE F	gCAgCAgCgCATgACggTAAAg	894	*sdr*E	[113]
	sdrE R	gTCgCCACCgCCAgTgTCATTA			
36	icaA F	TCAgACACTTgCTggCgCAgTC	936	*ica*A	[113]
	icaA R	TCACgATTCTCTCCCTCTCTgCCATT			
37	cap5 F	gTCAAAgATTATgTgATgCTACTgAg	361	*cap*5	[85]
	cap5 R	ACTTCgAATATAAACTTgAATCAAATgTTATA CAg			
38	cap8 F	gCCTTATgTTAggTgATAAACC	173	*cap*8	[85]
	cap8 R	ggAAAAACACTATCATAgCAgg			
39	sak F	AAggCgATgACgCgAgTTAT	210	*sak*	[86]
	sak R	gCgCTTggATCTAATTCAAC			
40	NUC-F166	AgTTCAgCAAATgCATCACA	400	*nuc*	[166]
	NUC-R565	TAgCCAAgCCTTgACgAACT			
41	COA-F2591	CCgCTTCAACTTCAgCCTAC	204	*coa*	[166]
	COA-R2794	TTAggTgCTACAggggCAAT			
42	Chp-F	TTTACTTTTgAACCgTTTCCTAC	350	*chp*	[86]
	Chp-R	CgTCCTgAATTCTTAgTATgCATATTCATTAg			
43	Scn-F	AgCACAAgCTTgCCAACATCg	180	*scn*	[86]
	Scn-R	TTAATATTTACTTTTTAgTgC			
44	hla F	CTgATTACTATCCAAgAAATTCgATTg	209	*hla*	[167]
	hla R	CTTTCCAgCCTACTTTTTTATCAgT			
45	hlb-F	gTgCACTTACTgACAATAgTgC	309	*hlb*	[167]
	hlb-R	gTTgATgAgTAgCTACCTTCAgT			
46	hld F	AAgAATTTTTATCTTAATTAAggAAggAgTg	111	*hld*	[167]
	hld R	TTAgTgAATTTgTTCACTgTgTCgA			
47	hlg F	TTggCTggggAgTTgAAgCACA	306	*hlg*	[113]
	hlg R	CgCCTgCCCAgTAgAAgCCATT			
48	hlgvF	gACATAgAgTCCATAATgCATTYgT	390	*hlg* variant	[167]
	hlgvR	ATAgTCATTAggATTAggTTTCACAAAg			
49	eta F	CgCTgCggACATTCCTACATgg	676	*eta*	[113]
	eta R	TACATgCCCgCCACTTgCTTgT			
50	etb F	gAAgCAgCCAAAAACCCATCgAA	419	*etb*	[113]
	etb R	TgTTgTCCgCCTTTACCACTgTgAA			

Table 2.2 cont. PCR primers used during this study.

51	tst F	AgCCCTgCTTTTACAAAAggggAAAA	306	*tst*	[113]
	tst R	CCAATAACCACCCgTTTTATCgCTTg			
52	SEA-F1170	TAAggAggTggTgCCTATgg	180	*sea*	[166]
	SEA-R1349	CATCgAAACCAgCCAAAgTT			
53	seb-F	gACATgATgCCTgCACCAggAgA	355	*seb*	[113]
	seb-R	AACAAATCgTTAAAAACggCgACACAg			
54	SEC-F97	ACCAgACCCTATgCCAgATg	371	*sec*	[166]
	SEC-R467	TCCCATTATCAAAgTggTTTCC			
55	SED-F578	TCAATTCAAAAgAAATggCTCA	339	*sed*	[166]
	SED-R916	TTTTTCCgCgCTgTATTTTT			
56	see-F	TgCCCTAACgTTgACAACAAgTCCA	532	*see*	[113]
	see-R	TCCgTgTAAATAATgCCTTgCCTgAA			
57	SEG-F322	CCACCTgTTgAAggAAgAgg	432	*seg*	[166]
	SEG-R753	TgCAgAACCATCAAACTCgT			
58	SEH-F260	TCACATCATATgCgAAAgCAg	463	*seh*	[166]
	SEH-R722	TCggACAATATTTTTCTgATCTTT			
59	SEI-F71	CTCAAggTgATATTggTgTAgg	529	*sei*	[166]
	SEI-R637	CAggCAgTCCATCTCCTgTA			
60	SEJ-F349	ggTTTTCAATgTTCTggTggT	306	*sej*	[166]
	SEJ-R654	AACCAACggTTCTTTTgAgg			
61	SEL-F158	CACCAgAATCACACCgCTTA	240	*sel*	[166]
	SEL-R397	CTgTTTgATgCTTgCCATTg			
62	sem-F	CTATTAATCTTTgggTTAATggAgAAC	300	*sem*	[167]
	sem-R	TTCAgTTTCgACAgTTTTgTTgTCAT			
63	sen-F	ATgAgATTgTTCTACATAgCTgCAAT	680	*sen*	[167]
	sen-R	AACTCTgCTCCCACTgAAC			
64	seo-F	AgTTTgTgTAAgAAgTCAAgTgTAgA	180	*seo*	[167]
	seo-R	ATCTTTAAATTCAgCAgATATTCCATCTAAC			
65	sep-F	AATCATAACCAACCGAATCA	520	*sep*	[86]
	sep-R	TCATAATGGAAGTGCTATAA			

2.3.16 Control strains used during the study:

Table 2.3 *S. aureus* isolates used as controls for various assays, excluding virulence PCRs.

Strain	Application	Other designation	Reference
NCTC8325-4	PFGE size reference standard	-	[68, 142]
ATCC BAA-38	SCC*mec* I	E2125	[29]
ATCC 1681	SCC*mec* II, *agr* II	HFH-29994	www.atcc.org; [160]
ATCC BAA-39	SCC*mec* III	HUSA304	[29]
MW2	SCC*mec* IV; PVL +	ATCC BAA-1707	[29, 65]
ATCC 1688	SCC*mec* V	HFH-30032	www.atcc.org
ATCC BAA-42	SCC*mec* VI	HDE288	[29]
ATCC 1680	*agr* I	HFH-29568	www.atcc.org
ATCC 1683	*agr* III	HFH-30364	www.atcc.org
THW-101	*agr* IV	-	This study

Table 2.4 Control isolates used for PCR investigation of virulence factors and PCR annealing temperatures.

Gene	Name	Control isolate	T_m (°C)	Reference
*fnb*A	Fibronectin binding protein A	COL	56	This study
*fnb*B	Fibronectin binding protein B	COL	60	This study
*clf*A	Clumping factor A	COL/ NCTC8325-4	56	This study
*clf*B	Clumping factor B	Newman	55	This study
Eap	Extracellular adherence protein	Newman	56	This study
cna	Collagen binding protein	ATCC25923	64	This study
*sdr*C	Serine-aspartate repeat protein C	Newman	64	This study
*sdr*D	Serine-aspartate repeat protein D	Newman	64	This study
*sdr*E	Serine-aspartate repeat protein E	Newman	64	This study
*ica*A	Biofilm cluster	NCTC8235-4	66	This study
*cap*5	Capsular polysaccharide 5	NCTC8325-4	60	This study
*cap*8	Capsular polysaccharide 8	MW2	58	This study
sak	Staphylokinase	NCTC8325-4	58	This study

Table 2.4 cont. Control isolates used for PCR investigation of virulence factors and PCR annealing temperatures.

coa	Coagulase	ATCC23235	56	This study
nuc	Nuclease	ATCC23235	56	This study
chp	Chemotaxis inhibitory protein	ATCC BAA38	54	This study
scn	Staphylococcal complement inhibitor	ATCC 23235	45	This study
hla	α toxin	NCTC8325-4	56	This study
hlb	β toxin	6850	56	This study
hld	δ toxin	NCTC8325-4	56	This study
hlg	γ toxin	NCTC8325-4	56	This study
hlg-variant	γ toxin (variant)	LAC	59	This study
eta	Exfoliative toxin A	ATCC 1861	68	This study
etb	Exfoliative toxin B	ATCC 1861	60	This study
tst	Toxic shock syndrome toxin-1	ATCC 700699	60	This study
sea	Staphylococcal enterotoxin A	ATCC 700699	56	This study
seb	Staphylococcal enterotoxin B	ATCC BAA38	66	This study
sec	Staphylococcal enterotoxin C	ATCC 700699	56	This study
sed	Staphylococcal enterotoxin D	ATCC23235	56	This study
see	Staphylococcal enterotoxin E	ATCC 700699	60	This study
seg	Staphylococcal enterotoxin G	ATCC 19095/ ATCC 23235	64	This study
seh	Staphylococcal enterotoxin H	ATCC19095	58	This study
sei	Staphylococcal enterotoxin I	ATCC 700699	66	This study
sej	Staphylococcal enterotoxin J	ATCC 19095	64	This study
sel	Staphylococcal enterotoxin L	ATCC 23235	64	This study
sem	Staphylococcal enterotoxin M	ATCC 19095	52	This study
sen	Staphylococcal enterotoxin N	ATCC19095	54	This study
seo	Staphylococcal enterotoxin O	ATCC19095	54	This study
sep	Staphylococcal enterotoxin P	THW-17	45	This study

2.3.17 Composition of media, buffers and solutions:

Table 2.5 Composition of media used.

Media	Composition
5% Blood agar plate	15 g pancreatic digest of casein; 15 g papaic digest of soy meal; 5 g NaCl; 15 g agar; Bring to volume of 1 l Autoclave pH to 7.3 Cool to 45-50 °C; add 5% (v/v) sterile defibrinated blood; mix
BHI broth	17.5 g brain heart extract; 10 g peptone; 2 g glucose; 5 g NaCl; 2.5 g disodium hydrogen phosphate Bring to volume of 1 l Autoclave pH 7.4

Table 2.6 Composition of buffers and solutions used.

Buffer/Solution	Composition
TE buffer	10 mM Tris-HCl; 1 mM EDTA (pH 8)
EC lysis buffer	6 mM Tris-HCl; 1 M NaCl; 100 mM EDTA; 0.5% Brij-58; 0.2% Na-deoxycholate; 0.5% Na-lauroylsarcosine
10× TBE	0.89 M Tris; 0.89 M Boric acid; 20 mM EDTA
Proteinase K buffer	0.1 M Tris (pH 7.5)
50× TAE	2 M Tris; 50 mM EDTA; 2 M glacial acetic acid (pH 7.6)

2.4 Results

2.4.1 Sample collection:

367 consecutive, non-duplicate *S. aureus* isolates were collected from the same number of patients over a period of one year, from 10th September 2009 till 6th September 2010.

2.4.2 Methicillin-resistance:

56 (15.3%) of the isolates collected displayed phenotypic resistance to methicillin by automated susceptibility testing using the Vitek II system or by the disc-diffusion method[161] and were recorded as MRSA isolates.

2.4.3 Patient demographics:

28.9% (n=106) of the isolates were also classified as paediatric cases (newborn to 12 years), while 71.1% (n=261) of the isolates were classified as adult cases (13 years and older).

59.4% (n=218) of the isolates were collected from males, 40.3% (n=148) were collected from females and 0.3% (n=1) from a post-mortem sample of a patient with unknown gender. The mean age was 30 years and ranged from 3 days to 87 years.

23.2% (n=85) of the isolates were collected from persons with a known HIV status, of which 24.7% (n=21) of these isolates were collected from HIV+ persons, representing 5.7% of the whole collection. A break-down of HIV status according to methicillin-resistance is given in Figure 2.2.

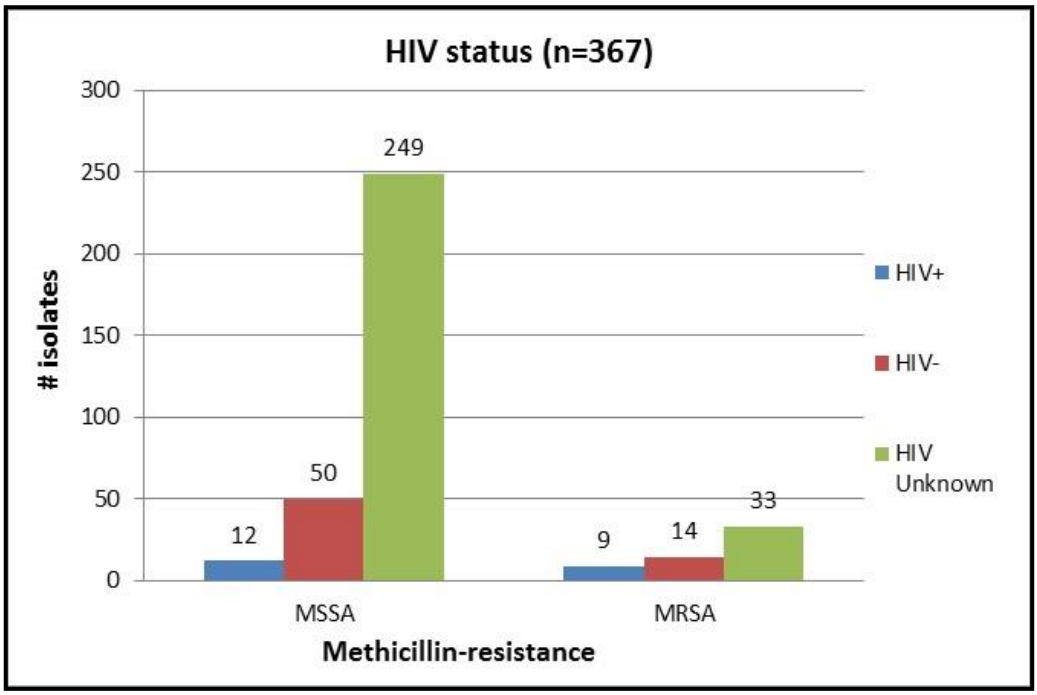


Figure 2.2 The distribution of isolates collected according to patient HIV status and methicillin-resistance.

2.4.4 Clinical sources of collected isolates:

95.9% (n=352) of the isolates were collected from known clinical sources. According to the clinical information provided by the requesting clinician, the isolates could be categorised as originating from 14 infection sources: skin and soft tissue (n=201); bone and joint (n=56); respiratory tract (n=28); prosthetic devices (n=21); eye (n=10); urinary tract (n=8); ear, nose and throat (n=6); intravascular devices (n=6); post mortem (n=4); deep seated abscesses (n=4); abdominal (n=3); cardiac (n=2); obstetrics and gynaecology (n=2); and dental (n=1). The remaining 4.1% (n=15) of the isolates were collected from unknown sources. Two of the post-mortem isolates were taken from lungs and one from the heart. A break-down of the number of isolates collected per source is provided in Figure 2.3.

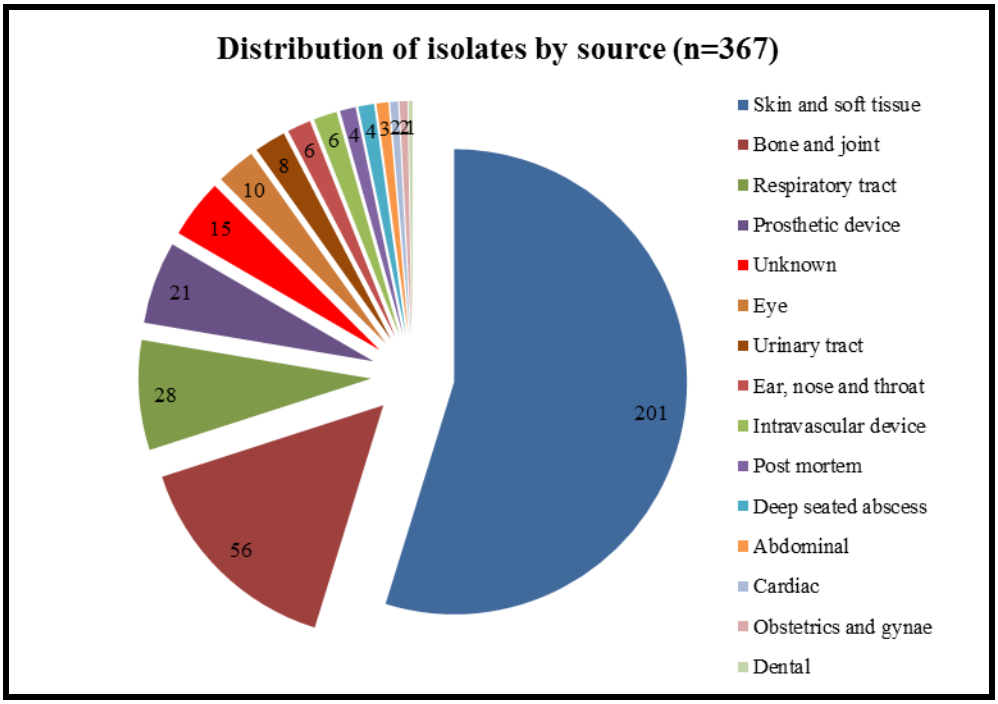


Figure 2.3 The distribution of isolates collected according to source.

2.4.5 Macro-restriction analyses (MRA) with pulsed-field gel electrophoresis (PFGE):

99.7% (n=366) of the collected 367 isolates were typeable by *Sma*I restriction analysis and resulted in the generation of 269 unique PFGE types, which could be grouped into 31 PFGE clones and 21 singletons at a 80% similarity cut-off on the UPGMA dendogram. The clones were further classified based on the number of isolates and unique PFGE types as major (> 10 isolates and PFGE types), intermediate (4-9 isolates and PFGE types) or minor (2-3 isolates and PFGE types).

THW-C was identified as the dominant clone and was composed of 17.2% (n=63) predominantly MSSA isolates. THW-A and THW-V had 12.6% (n=46) MSSA isolates each. The dominant MRSA clone identified was THW-O and was composed of 23.2% (n=13) MRSA isolates (3.5% total isolates).

69.4 % (n=254) of the typeable isolates grouped into eight major clones, 16.9% (n=62) grouped into ten intermediate clones and 7.9% (n=29) grouped into 13 minor clones. Please refer to Table 2.7 for the number of isolates and PFGE types, as well as the methicillin susceptibilities of each clone identified and Figure 2.4 for a collapsed version of the UPGMA dendogram representing the whole population.

Table 2.7 PFGE clones identified during this study by *Sma*I digestion. The clones were classified as major, intermediate or minor based on the number of isolates and unique PFGE types.

Name	Status	# isolates	# PFGE types/patterns	Methicillin-resistance
THW-A	Major	46	28	MSSA
THW-C		63	27	Mixed (92% MSSA)
THW-E		16	12	MSSA
THW-O		13	11	Mixed (92% MRSA)
THW-S		16	14	MSSA
THW-V		46	31	MSSA
THW-Y		17	15	MSSA
THW-EE		37	27	MSSA
THW-B	Intermediate	6	5	MRSA
THW-G		7	5	MSSA
THW-J		6	6	Mixed (67% MSSA)
THW-L		6	6	MRSA
THW-N		8	8	MRSA
THW-W		5	4	MSSA
THW-X		5	5	MSSA
THW-Z		6	5	MSSA
THW-AA		9	8	MRSA
THW-BB		4	4	Mixed (50% MSSA)
THW-D	Minor	3	2	MSSA
THW-F		3	3	MSSA
THW-H		2	2	MSSA
THW-I		3	2	Mixed (67% MSSA)
THW-K		2	2	MSSA
THW-M		2	2	MSSA

Table 2.7 cont. PFGE clones identified during this study by *Sma*I digestion. The clones were classified as major, intermediate or minor based on the number of isolates and unique PFGE types.

THW-P	Minor	2	2	MRSA
THW-Q		2	2	MSSA
THW-R		2	2	MSSA
THW-T		2	2	MSSA
THW-U		2	2	MSSA
THW-CC		2	2	MSSA
THW-DD		2	2	MSSA
ST	Singletons	21	21	Mixed (86% MSSA)

= number of

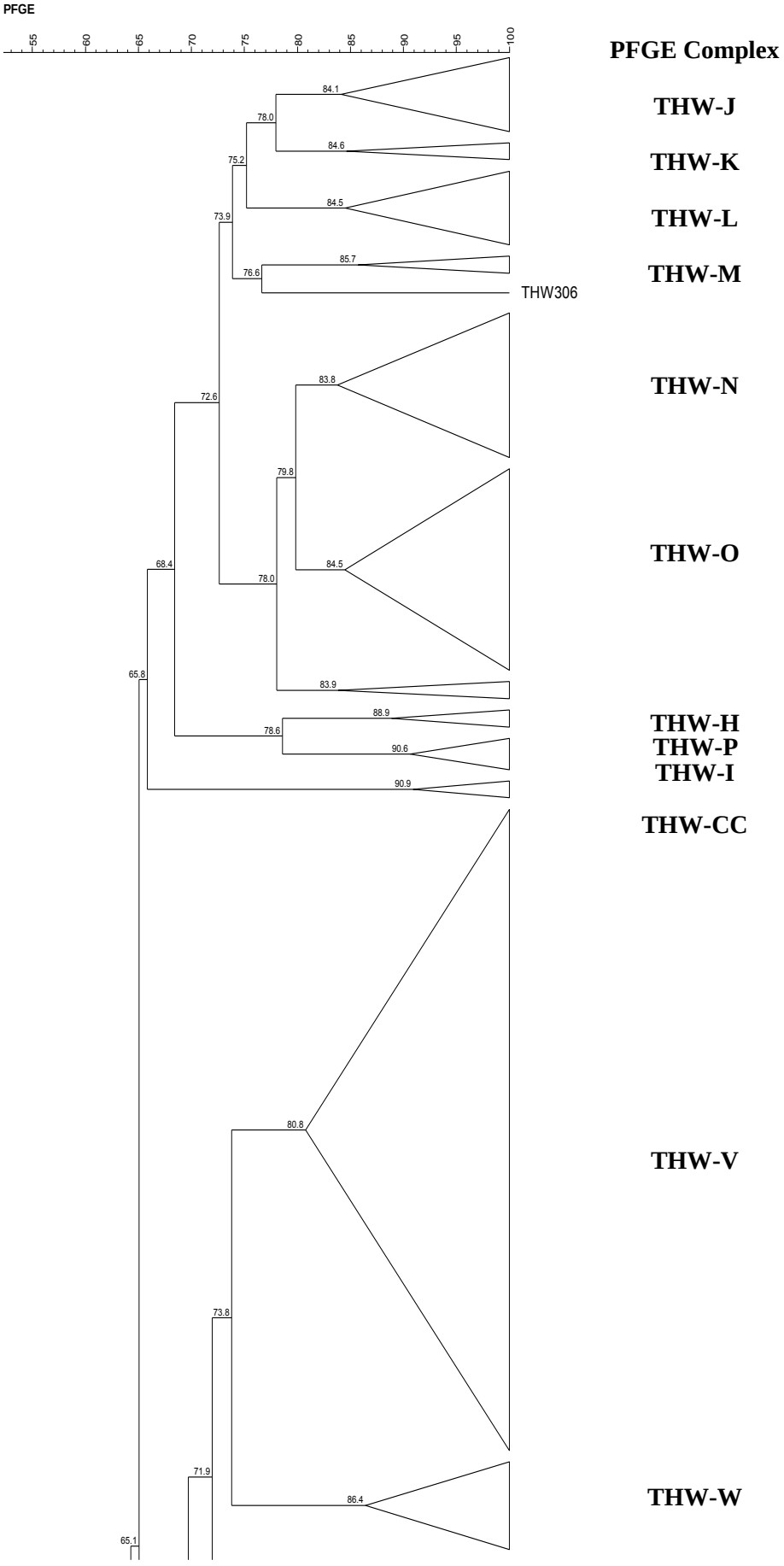


Figure 2.4 cont. Collapsed UPGMA dendogram of the 366 typeable *S. aureus* isolates.

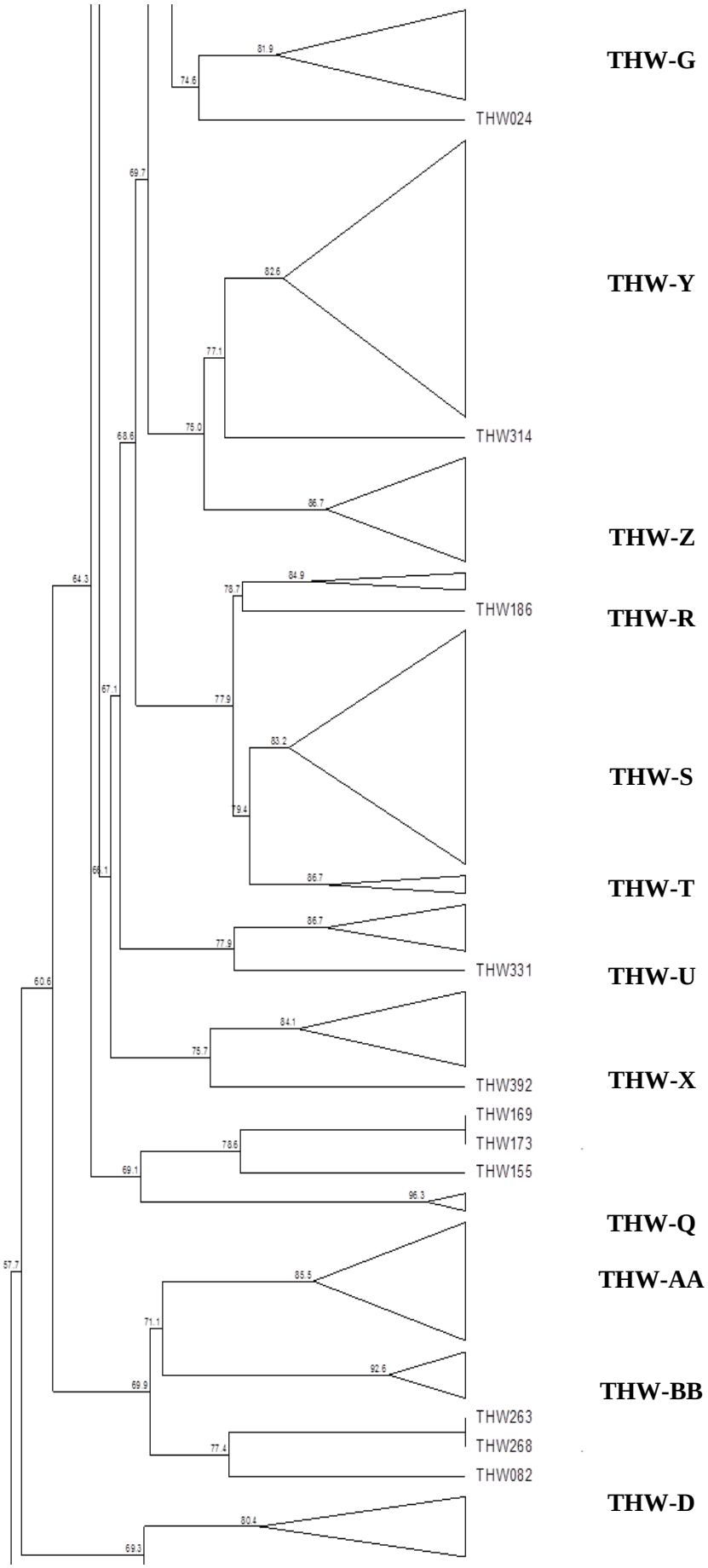


Figure 2.4 cont. Collapsed UPGMA dendogram of the 366 typeable *S. aureus* isolates.

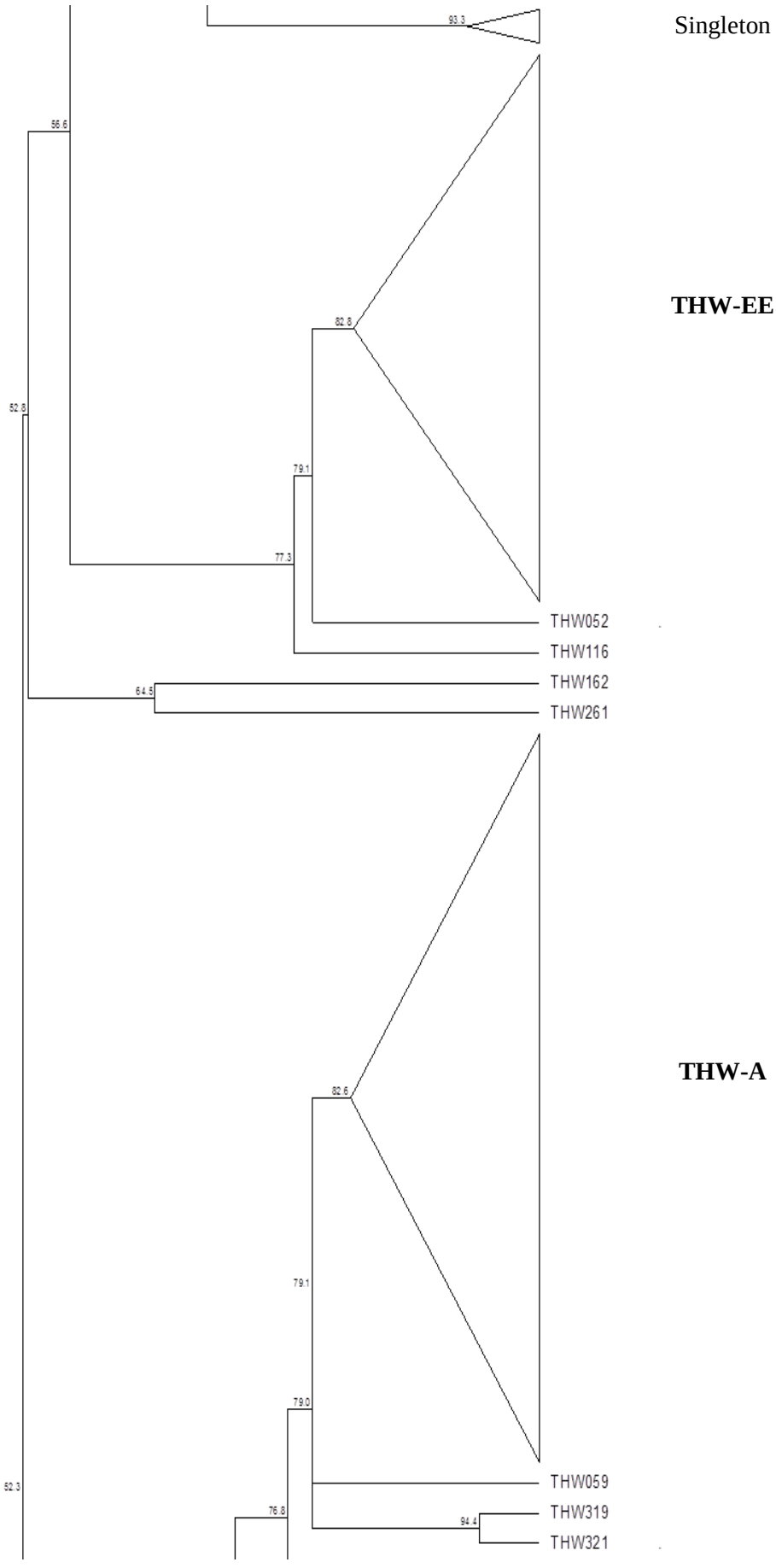


Figure 2.4 cont. Collapsed UPGMA dendogram of the 366 typeable *S. aureus* isolates.

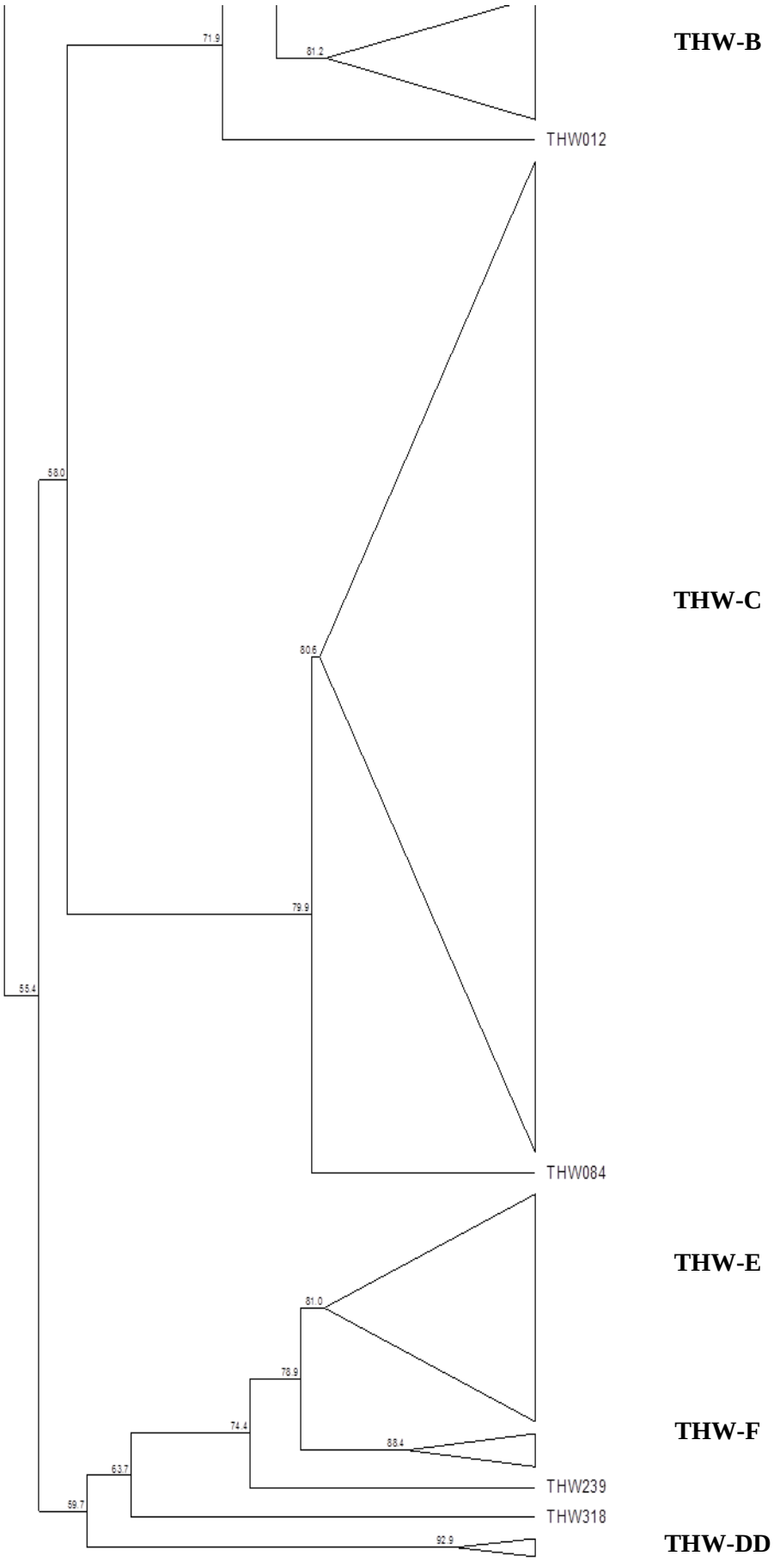


Figure 2.4 cont. Collapsed UPGMA dendogram of the 366 typeable *S. aureus* isolates.

2.4.6 *spa* typing:

The *spa* types of 99.7% (n=366) isolates were determined and one isolate was non-typeable (NT). 127 unique *spa* types were identified. Thirty five of these *spa* types were identified as novel types, of which one was identified as novel due to the identification of a novel repeat unit. 78 *spa* types (21.3%, n=78) represented single isolates in this study. All isolates possessed a single *spa* type.

The dominant *spa* type was t891 (13.9%, n=51), followed by t002 (5.2%, n=19), t318 (4.9%, n=18), t317 (4.4%, n=16) and t174 (4.1%, n=15).

Using the BURP algorithm of the RIDOM StaphType software, the 127 *spa* types were clustered into 16 *spa*-CCs (Figure 2.5; Table 2.8). *spa* types from 14 (3.8%) isolates were classified as singletons, while eight *spa* types from seven (1.9%) isolates were excluded from the analyses as they were too short. *Spa*-CC891 was identified as the dominant *spa*-CC, comprising 17.5% (n=64) of the isolates. *Spa*-CC021 contained 16.9% of the isolates (n=62), followed by *spa*-CC 064, which contained 15.8% of the isolates (n=58) (Table 2.8).

2.4.7 SCC*mec* typing:

All MRSA isolates (n=56) were screened to determine which variant of the SCC*mec* element was present in each isolate.

SCC*mec* type IV was identified as the dominant variant in 53.6% of the isolates (n=30) and was subtyped as SCC*mec* type IV (n=28) and SCC*mec* type IV-E (n=2). SCC*mec* type I was identified in 16.1% of the isolates (n=9), followed by SCC*mec* type II in 10.7% of the isolates (n=6) and SCC*mec* types III and V in 8.9% of the isolates each (n=5). One isolate was non-typeable and yielded no amplification pattern, except for the *mecA* internal control.

Table 2.8 *spa*-CC identified during this study using the BURP algorithm of RIDOM StaphType®.

***spa*-CC**	***spa* types**	**# isolates**	**Number *spa* types**	**Methicillin-resistance**
spa-CC891	020, 032, 891, 1972, 2251, 6572*, 6613*, 6669*,6717*, 7025*, 7027*, 7069*, 7181*	64	13	Mixed
spa-CC021	012, 021, 030, 037, 122, 238, 275, 318, 338, 433, 964, 1152, 1848, 3724, 4600, 5542, 6539*, 7068*, 7183*, 7209*	62	20	Mixed
spa-CC064	008. 064, 304, 701, 1257, 1443, 1476, 1779, 2179, 2360, 6538*, 6541*, 6612*, 7070*	58	14	Mixed
spa-CC002	002, 045, 071, 311, 3794, 5300, 6100, 6571*, 6614*, 7021*, 7026*	34	11	Mixed
spa-CC015	015, 050, 073, 465, 487, 833, 1156, 1510, 3554, 5381, 7022*, 7023*, 7036*, 7182*	33	14	MSSA
spa-CC084	084, 085, 346, 853, 1877, 3366, 7071	20	7	MSSA
spa-CCNF:16	314, 317	18	2	MSSA
spa-CCNF:14	174, 6570	14	2	MSSA
spa-CC267	267, 934, 1839	8	3	MSSA
spa-CC1597	078, 1054, 1597, 7020*, 7098*	8	5	MSSA
spa-CC888	160, 888, 6537	6	3	MSSA
spa-CC148	148, 324, 3092	5	3	MSSA
spa-CCNF:13	164, 881	5	2	MSSA
spa-CC186	186, 729, 1591, 5351	4	4	Mixed
spa-CC191/100	100, 191, 3463	3	3	MSSA
spa-CCNF:15	1931, 7072	2	2	MSSA
Singletons	111, 159, 189, 355, 3265, 4545, 6542*, 6569*, 7059*	14	9	Mixed
Excluded	026, 779, 870, 1200, 5633, 7024*, 7096*	8	7	Mixed

*= Novel *spa* type; Mixed = methicillin-susceptible and resistant

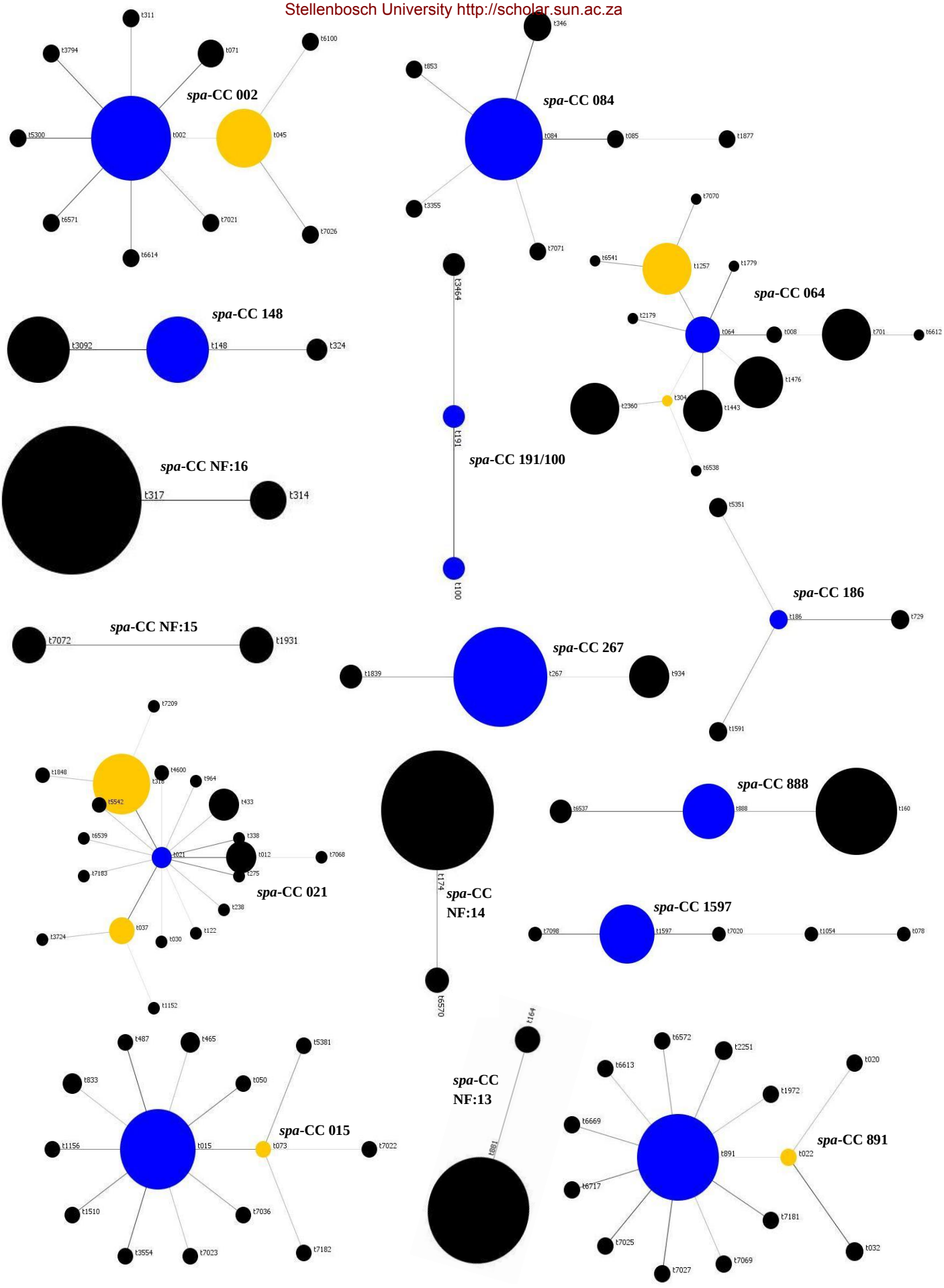


Figure 2.5 Graphical representations of identified *spa*-clonal complexes. Blue circles indicate founder types and yellow circles indicate sub-founders.

2.4.8 *agr* typing:

The *agr* type of all isolates were determined and all four *agr* types were identified, where *agr* type I was the dominant type, identified in 54.2% of the isolates (n=199). *agr* type III was identified in 21.8% of the isolates (n=80), followed by *agr* type II, identified in 18.5% of the isolates (n=68) and *agr* type IV, identified in 5.4% of the isolates (n=20).

agr type I was the dominant in both MSSA and MRSA isolates, accounting for 51.4% and 69.6% of the isolates respectively, while *agr* type IV was only identified in MSSA isolates. The *agr* distribution according to methicillin-resistance can be seen in Figure 2.6 below.

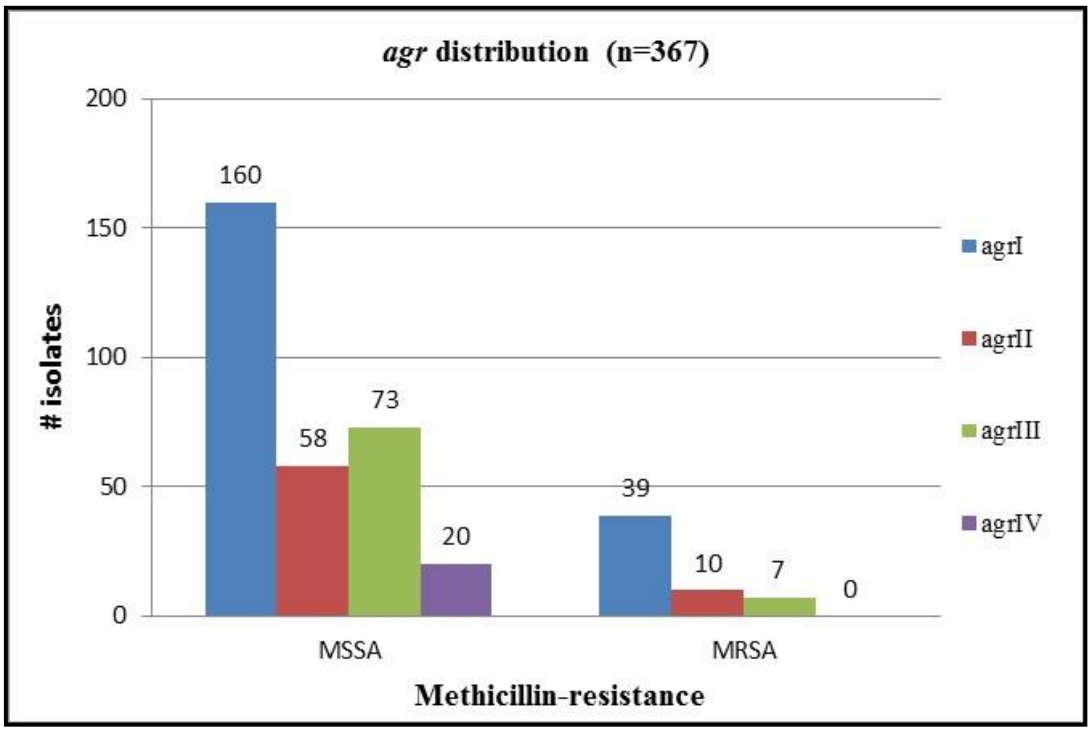


Figure 2.6 *agr* distribution according to methicillin-resistance.

2.4.9 PCR detection of Panton-Valentine leukocidin (PVL):

All isolates were screened for the presence of the *luk*S/F genes and 43.6% (n=160) of the isolates tested positive. 49.8% of the MSSA isolates (n=155) were PCR +, while only 9.9% (n=5) of the MRSA isolates tested positive (Figure 2.7).

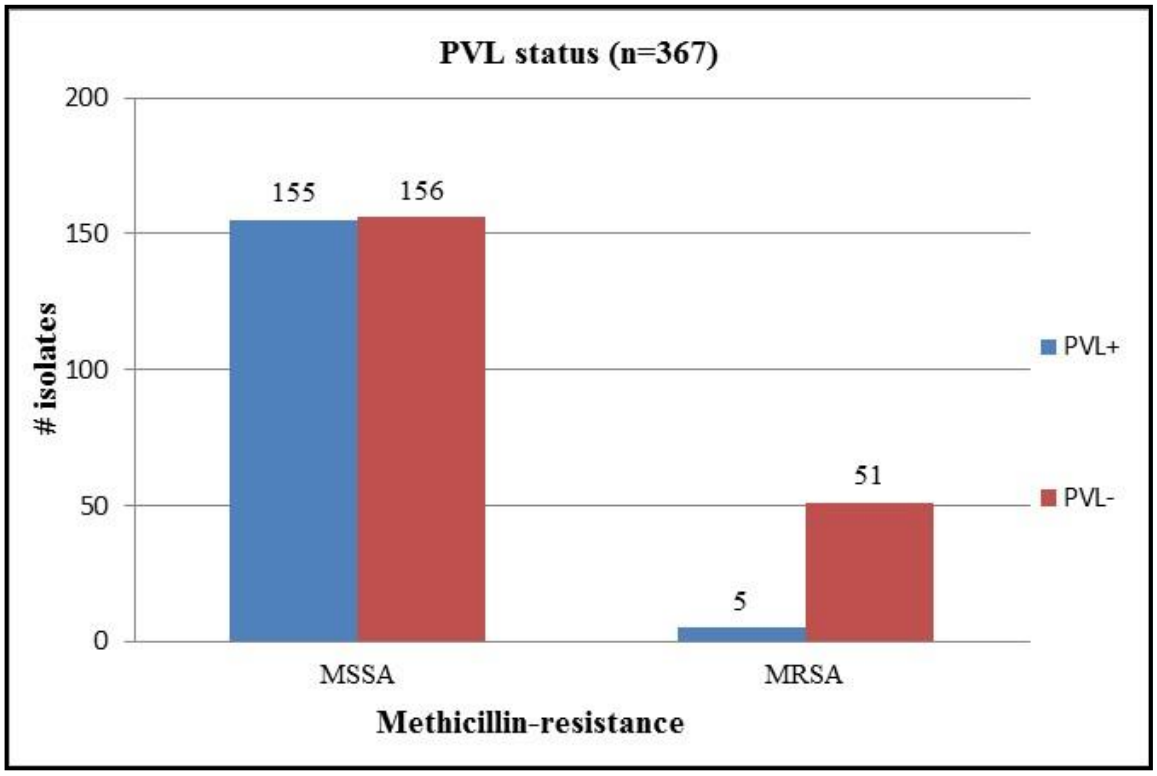


Figure 2.7 *lukS/F* PCR results according to methicillin-resistance.

2.4.10 *Apa*I MRA and PFGE of clone THW-V:

After all typing data acquired was combined, it was noticed that a good correlation was achieved by the results of the various typing techniques. All isolates from all individual PFGE clones grouped into single *spa*-CCs and possessed the same *agr* variant respectively. This was not the case for clone THW-V, where four *spa*-CCs were observed and three *agr* variants. It was decided to repeat the PFGE analyses, using *Apa*I restriction enzyme (RE).

Restriction using the *Apa*I RE clearly showed the presence of three clusters using an 80% cut-off (Figure 2.8), which correlated with the *agr* typing and were given a numerical number combined with the previously assigned alphabetical name, thus giving THW-V1, THW-V2 and THW-V3 (Table 2.9).

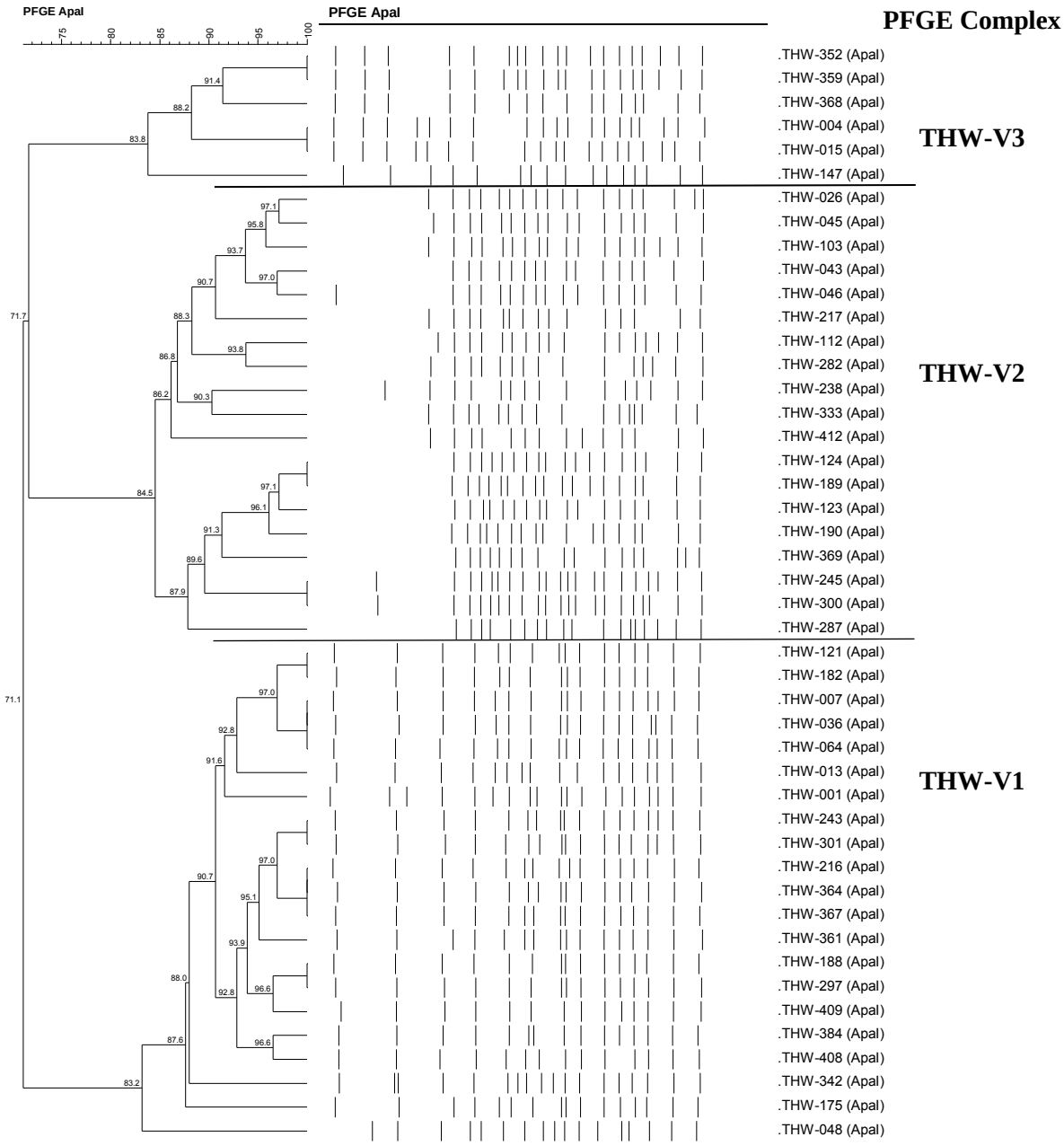


Figure 2.8 Clustering of isolates from clone THW-V by *Apa*I macro-restriction.

Table 2.9 *Apa*I PFGE clones identified from *Sma*I clone THW-V, with supporting data from *spa*, *agr* and SCC*mec* typing.

PFGE	# isolates	Status	*spa*-CC	*agr*	PVL
THW-V1	21	Major	64	I	5% +
THW-V2	19	Major	NF:14	III	47% +
THW-V3	6	Intermediate	888	II	17% +

2.4.11 Multi-locus sequence typing (MLST):

Based on the molecular data acquired by the previously discussed typing techniques, a representative isolate was randomly selected for each major and intermediate PFGE clone (*Sma*I and *Apa*I digestions). Two isolates were selected for THW-C as this clone was mixed in resistance, one MSSA and one MRSA isolate. 19 MLST STs were identified from 21 isolates, which clustered into two MLST CC and 14 singletons using a similarity of 6/7 loci. To group singletons into clonal complexes, eBURST analyses was performed again, using all previous MLST STs submitted to the database and MLST STs identified during this study. This allowed us to group the MLST STs into four CCs (CC30, CC8, CC15 and CC5) and seven singletons (ST22, ST121, ST45, ST6, ST12, ST97 and ST88) (Table 2.10).

2.4.12 Combined molecular data:

A good correlation was noticed after combination of data obtained by the various typing techniques. All major (9 isolates selected) or intermediate PFGE clones (11 isolates selected) had a unique *agr* type, *spa*-CC and were predominantly or complete PVL+ or PVL-. Only four clones had both methicillin susceptible and resistant isolates: THW-C, THW-J, THW-O and THW-BB. All PFGE clones had a unique MLST ST, except for ST612 which was identified in THW-N and THW-O. *Spa*-CCs were divided into numerous clones by both PFGE and MLST, i.e. *spa*-CC064 was identified in THW A-ST1685-MSSA, THW B-ST36-II and THW L-ST239-II. A unique SCC*mec* type was present in all MRSA isolates with-in the same clone, except for THW-L where one isolate was non-typeable, and THW-C, where both SCC*mec* types IV (PVL-) and V (PVL +) were

identified. A correlation between MLST, *spa* and *agr* typing data was only achieved through *Apa*I MRA for PFGE clustering of clone THW-V.

Table 2.10 MLST CC identified and their respective PFGE clone.

MLST		Methicillin-resistance (SCC*mec* type)	PFGE	
CC	**ST**		**Name**	**n**
22	22	MSSA	THW-C	63
30	1865	MSSA	THW-A	46
	36	II	THW-B	6
8	8	MSSA/V	THW-J	6
	612	IV	THW-N	8
			THW-O	13
	239	III/NT	THW-L	6
	1862	MSSA	THW-G	7
15	1	MSSA	THW-V2	19
	15	MSSA	THW-S	16
	188	MSSA	THW-X	4
45	45	MSSA	THW-EE	37
5	5	I	THW-AA	9
	1863	MSSA	THW-Y	17
	1864	MSSA	THW-Z	6
6	6	MSSA	THW-V1	21
121	121	MSSA	THW-E	16
12	12	MSSA	THW-V3	6
97	97	MSSA	THW-W	5
88	88	MSSA/IV	THW-BB	4

NT = non-typeable

Regarding clones THW-C, THW-J and THW-O, it was noticed that a corresponding MSSA strain was present for a MRSA strain with the same *spa* type, which might suggest local SCC*mec* acquisition events. Please refer to Table 2.11 below for a breakdown of the typing data according to PFGE classification.

Table 2.11 PFGE clones identified, with supporting data from other typing techniques.

PFGE	# isolates*	Status	MLST		*spa*-CC	SCC*mec*	*agr*	PVL ± (%)
			ST	CC				
THW-A	46	Major	1865	30	21	MSSA	III	83% +
THW-B	6	Intermediate	36	30	21	II	III	-
THW-C	63	Major	22	22	891	59 MSSA I *mec* V 4 *mec* IV	I	+ + -
THW-D	5	Minor	ND	ND	NF:13	MSSA	I	-
THW-E	16	Major	121	121	NF:16	MSSA	IV	94% +
THW-F	3	Minor	ND	ND	NF:16	MSSA	IV	+
THW-G	7	Intermediate	1862	8	1597	MSSA	I	43% +
THW-H	2	Minor	ND	ND	64	MSSA	I	50% +
THW-I	3	Minor	ND	ND	148	2 MSSA 1 *mec* V	I	- -
THW-J	6	Intermediate	8	8	64	4 MSSA 2 *mec* V	I	25% + -
THW-K	2	Minor	ND	ND	64	MSSA	I	50% +
THW-L	6	Intermediate	239	8	21	5 III 1 NT	I	-
THW-M	2	Minor	ND	ND	64	MSSA	I	-
THW-N	8	Intermediate	612	8	64	IV	I	-
THW-O	13	Major	612	8	64	1 MSSA 12 *mec* IV	I	- 8% +
THW-P	2	Minor	ND	ND	64	IV	I	-
THW-Q	2	Minor	ND	ND	267	MSSA	III	+
THW-R	2	Minor	ND	ND	84	MSSA	II	-
THW-S	16	Major	15	15	84	MSSA	II	14% +
THW-T	2	Minor	ND	ND	84	MSSA	II	-
THW-U	2	Minor	ND	ND	191/100	MSSA	II	50% +
THW-V	6 (V3) 14 (V2) 18 (V1) 2	Intermediate Major Major Minor	12 1 6 ND	12 15 6 ND	888 NF:14 64 NF:15	MSSA	II III I III	17% + 36% + 6% + +

Table 2.11 cont. PFGE clones identified, with supporting data from other typing techniques.

THW-W	5	Intermediate	97	97	267	MSSA	I	-
THW-X	4	Intermediate	188	15	189	MSSA	I	25% +
THW-Y	17	Major	1863	5	2	MSSA	II	6% +
THW-Z	6	Intermediate	1864	5	2	MSSA	II	17% +
THW-AA	9	Intermediate	5	5	2	I	II	-
THW-BB	4	Intermediate	88	88	186	2 MSSA 2 *mec* IV	III	50 % + +
THW-CC	2	Minor	ND	ND	1597	MSSA	I	-
THW-DD	2	Minor	ND	ND	ST	MSSA	I	+
THW-EE	37	Major	45	45	15	MSSA	I	16% +

* = Isolates classified as "singletons" and "excluded *spa* types" using BURP analyses were excluded; ND = not determined

PFGE displayed the highest discriminatory index, while PFGE clustering using 80% as a cut-off value displayed the highest DI for clustering of individual isolates into clonal complexes. Please refer to Table 2.12-

Table 2.12 The discriminatory power of the typing techniques used during this study.

Technique	# strains included	# types/clonal complexes	DI	95% CI
PFGE	366	269	0.991	0.988-0.993
PFGE/80% clustering	366	31	0.918	0.905-0.931
***spa* typing**	366	127	0.965	0.956-0.974
***spa*/BURP**	366	16	0.890	0.877-0.902

2.4.13 Statistical analyses:

All major and intermediate clones were selected to investigate if any associations existed between clonality and the following categories: (a) methicillin-resistance (MRSA and/or MSSA); (b)

Paediatric and/or adult derived clones; (c) clones associated with a specific source/site of infections; (d) clones associated with a specific gender; and (e) clones associated with a specific PVL status, i.e. PVL+ and/or PVL- clones. All clones were selected to investigate if any association existed between clonality and HIV status.

A significant association was found between clonality and methicillin-resistance (p=0.0001) for all major clones and nine intermediate clones, as well as PVL status (p=0.0001) for all major and intermediate clones. No association was found between clonality and adult or paediatric infections (p=0.644) for all major clones, as well as for gender (p=0.092). A significant association was found between clonality and adult infections (p=0.001) for three intermediate clones, THW-B, THW-G and THW-J. An association between clonality and gender (p=0.04) was also identified for a single intermediate clone only, THW-W. THW-A, THW-C and THW-E were the only major clones for which an association was identified between clonality and source of infection (p=0.001), in this case skin and soft tissue infections (SSTI). A statistical trend was observed for the intermediate clones THW-B, THW-BB and THW-G, although this was not significant (p=0.07) (Table 2.13).

Regarding HIV status, only the minor clones THW-K and THW-P were found to be associated with an HIV+ status (p=0.0001). An association with HIV status was also found for clones THW-C and THW-O (p=0.0001), however, due to the large number of strains within these clonal complexes collected from patients with an unknown HIV status, we were unable to determine what the true correlation with HIV status is. It should be noted that the highest incidence of a positive HIV status was found in these two clones respectively, and THW-C was the dominant MSSA clone while THW-O was the dominant MRSA clone (Table 2.13).

2.4.14 Selection of representative isolates:

The main aim of the population analysis described in this first part of the study, was to select representative isolates for further analysis. Further analyses of the representative isolates focused on genotyping the presence of numerous virulence factors as well as investigating phenotypic host-pathogen interaction characteristics, such as cellular invasiveness and cell death induction.

As a representative of a specific clonal complex, we randomly selected an isolate from each major and intermediate PFGE clone identified using the “=rand()” function of MS Excel in “column 1” and copied the values obtained into “column 2”, and the values of “column 2” into column 3. The members of each clonal complex were then arranged based on the values of “column 3” from

smallest to largest. The isolate with the smallest number was selected. The isolates (n=20) were selected to ensure that they were representative of at least two categories each where statistical significance was found, except for clone THW V2-ST1-MSSA and THW Z-ST1864-MSSA.
A representative isolate was also selected from the two clones where an association was established with an HIV+ status, THW-K and THW-P [HIV + (ass.)]. As the highest incidence of an HIV+ status was found in the dominant MSSA clone, THW-C, as well as the dominant MRSA clone, THW-O, an isolate from all the patients with an HIV+ status of THW-C and THW-O was also selected [HIV+ (isol.)]. The MLST STs of these four isolates were also determined. The final isolate selected as a representative was the only MRSA isolate which possessed a non-typeable SCC*mec* element, THW L-ST239-Unknown. Please refer to Table 2.14 for a complete breakdown of the isolates selected for further analyses.

2.4.15 Virulence factor PCRs:

All 25 representative isolates tested positive for *fnb*A/B, *clf*A/B, *eap, coa, nuc* and *hld*, while *hla, hlg*-variant, *sak* and *sea* were identified in 20 or more isolates. The genes *tst, hlb, sec, seh, sej, sep* and *eta* were scarce, testing positive in only four or less isolates and *sed, sel* and *etb* were completely absent. The following genes were identified in a selected number of isolates: *cna* (n = 13), *sdr*D (n = 15), *sdr*E (n = 16), *ica*A (n = 16), *cap*5 (n = 14), *hlg* (n = 20), *scn* (n = 17), *sdr*C (n = 11), *cap*8 (n = 11), *chp* (n = 11), *seb* (n = 6), *see* (n = 11), *seg* (n = 9), *sei* (n = 12), *sem* (n = 10), *sen* (n = 11) and *seo* (n = 10).

Genotypically, we found the presence of numerous invasins (*fnb*A/B, *eap*) in all the isolates selected and tested. Virulence factors primarily involved in adhesion were also identified in all the selected isolates (*clf*A/B), while some adhesion factors were present in fairly high numbers (*cna, sdr)* together with *ica*A locus, associated with biofilm growth and persistence. Genes encoding proteins associated with cell death induction were very common (*hla, hlg, hlg*-variant), as were those associated with host immune evasion (*cap*5/8, *scn, chp*), while toxin genes were generally scarce.

Fifteen isolates tested positive for at least two *sdr* genes (associated with adherence), while either *cap5* or *cap8* was present in each isolate, but never both. At least 20 isolates tested positive for either *scn, chp, sak, sea* or *sep* (or a combination of these), genes commonly associated with an innate immune evasion cluster (IEC) carried on β-hemolysin-converting bacteriophages[86]. This

correlates well with the low prevalence of and expression of *hlb*, as it is known that these phages integrate into this gene[86]. *sem*, *sen* and *seo* were also identified as a cluster in nine isolates.

Please refer to Appendix A (Table A1) for a complete breakdown of the virulence factors identified in every isolate, and to Table 2.15 for the prevalence of each virulence factor.

Table 2.13 Statistical associations between clonality and the clinical/bacterial characteristics using the Fisher's exact test.

PFGE	MLST (ST:CC)	Status	Category (p-value)					
			Methicillin-resistance	Paediatric and/or adult	Source of infection	Gender	PVL	HIV
THW-A	1865:30	Major	**0.0001 (S)**	0.644	**0.0001**	0.092	**0.0001**	ND
THW-B	36:30	Intermediate	**0.0001 (R)**	**0.001 (adult)**	**0.007**	0.114	**0.0001**	ND
THW-C	22:22	Major	**0.0001 (S)**	0.644	**0.0001**	0.092	**0.0001**	**0.0001 (HIV status)**
THW-E	121:121	Major	**0.0001 (S)**	0.644	**0.0001**	0.092	**0.0001**	ND
THW-G	1862:8	Intermediate	**0.0001 (S)**	**0.001 (adult)**	**0.007**	0.114	0.095	ND
THW-J	8:8	Intermediate	**0.005**	**0.001 (adult)**	0.07	0.114	**0.0001**	ND
THW-K	8:8	Minor	ND	ND	ND	ND	ND	**0.0001 (HIV+ status)**
THW-L	239:8	Intermediate	**0.0001 (R)**	0.917	0.07	0.114	**0.0001**	ND
THW-N	612:8	Intermediate	**0.0001 (R)**	0.917	0.07	0.114	**0.0001**	ND
THW-O	612:8	Major	**0.0001 (R)**	0.644	0.07	0.092	**0.0001**	**0.0001 (HIV status)**
THW-P	612:8	Minor	ND	ND	ND	ND	ND	**0.0001 (HIV+ status)**
THW-S	15:15	Major	**0.0001 (S)**	0.644	0.07	0.092	**0.0001**	ND
THW-V	Various	Major	**0.0001 (S)**	0.644	0.07	0.092	**0.0001**	ND
THW-W	97:97	Intermediate	**0.0001 (S)**	0.917	0.07	**0.04 (M)**	**0.0001**	ND
THW-X	188:15	Intermediate	**0.0001 (S)**	0.917	0.07	0.114	**0.0001**	ND
THW-Y	1863:5	Major	**0.0001 (S)**	0.644	0.07	0.092	**0.0001**	ND
THW-Z	1864:5	Intermediate	**0.0001 (S)**	0.917	0.07	0.114	**0.0001**	ND
THW-AA	5:5	Intermediate	**0.0001 (R)**	0.917	0.07	0.114	**0.0001**	ND
THW-BB	88:88	Intermediate	**0.005**	0.917	**0.007**	0.114	**0.0001**	ND
THW-EE	45:45	Major	**0.0001 (S)**	0.644	0.07	0.092	**0.0001**	ND

F = female; M = male; ns = not significant; ND = not determined; ST = sequence type; CC = clonal complex; PVL = Panton-Valentine leukocidin; R = resistant; S = sensitive

Table 2.14 Strains selected as representative isolates for further analyses.

Isolate name	PFGE	Clinical data	Status	MLST ST	MLST CC	*spa*-CC	SCC*mec*	*agr*	PVL	Statistical association
THW38	THW-A	Lip abscess	Major	1865	30	21	NA	III	+	MSSA/PVL+/SSTI
THW382	THW-B	Laparotomy (day 15 post-operation)	Inter	36	30	21	II	III	-	MRSA/PVL-/Adults/SSTI
THW146	THW-C	Abscess left knee	Major	22	22	891	NA	I	+	MSSA/PVL+/SSTI
THW393	THW-C	Septic left tibia with external fixation	Major	22	22	891	NA	I	+	HIV+ (isol.)
THW366	THW-E	Chronic folliculitis	Major	121	121	NF:16	NA	IV	+	MSSA/PVL+/SSTI
THW262	THW-O	Pyrexia, specimen from intracostal drainage site	Major	612	8	64	IV	I	-	MRSA/PVL-
THW65	THW-G	Septic ORIF	Inter	1862	8	1597	NA	I	-	MSSA/Adults
THW271	THW-J	Sinus fungal infection	Inter	8	8	64	V	I	-	PVL-/Adults
THW81	THW-L	Sepsis, pin tract	Inter	239	8	21	III	I	-	MRSA/PVL-
THW70	THW-N	Sepsis, nosocomial MRSA	Inter	612	8	64	IV	I	-	MRSA/PVL-
THW195	THW-K	Impetigo	Minor	8	8	64	NA	I	-	HIV+ (ass.)
THW273	THW-P	Pus from hand	Minor	612	8	64	IV	I	-	HIV+ (ass.)
THW93	THW-O	Sepsis	Major	612	8	64	IV	I	-	HIV+ (isol.)
THW99	THW-L	Septic wound	Inter	239	8	21	UK	I	-	Non-typeable SCC*mec*
THW122	THW-S	Sepsis	Major	15	15	84	NA	II	-	MSSA/PVL-
THW412	THW-V2	No clinical data supplied	Major	1	15	NF:14	NA	III	-	MSSA
THW224	THX-X	Wound sepsis	Inter	188	15	EX	NA	I	-	MSSA/PVL-

Table 2.14 cont. Strains selected as representative isolates for further analyses.

Isolate name	PFGE	Clinical data	Status	MLST ST	MLST CC	*spa*-CC	SCC*mec*	*agr*	PVL	Statistical association
THW17	THW-Y	Wound sepsis	Major	1863	5	2	NA	II	-	MSSA/PVL-
THW235	THW-Z	Measles	Inter	1864	5	2	NA	II	+	MSSA
THW264	THW-AA	Conjunctivitis	Inter	5	5	2	I	II	-	MRSA/PVL-
THW64	THW-V1	Suspect TB, pericardial fluid	Major	6	6	64	NA	I	-	MSSA/PVL-
THW356	THW-EE	Hip abscess	Major	45	45	EX	NA	I	-	MSSA/PVL-
THW368	THW-V3	Wound infection	Inter	12	12	888	NA	II	+	MSSA/PVL+
THW241	THW-W	ESRF	Inter	97	97	267	NA	I	-	MSSA/PVL-/Males
THW255	THW-BB	Infected leg	Inter	88	88	186	IV	III	+	PVL+/SSTI

ass. = associated; isol. = isolated; ESRF = end-stage renal failure; ORIF = open reduction and internal fixation; Inter = intermediate; NA = not applicable; Ex = excluded; SSTI = skin and soft tissue

The genes *sdr*C and *sei* were the only virulence factors associated with both methicillin-resistance and clonality, where both *sdrC* and *sei* were associated with MRSA isolates of CC5 and CC8. The gene *see* was associated with methicillin-resistance and identified only in MRSA isolates. The genes *cap5* and *cap8* were associated with different clonal complexes, where *cap5* was associated with CC5, CC8 and CC22 isolates, while *cap*8 was associated with CC15 and CC30 isolates. The genes *seg*, *sem* and *seo* were also associated with the same CCs, namely CC5, CC22 and CC30. The gene *sdr*E displayed a broad association, and included isolates from CC5, CC8, CC15 and CC22, while *hlgv* was associated with CC5, CC8 and CC15 isolates. Please refer to Table 2.16 for the associations of individual virulence factors with methicillin-resistance and/or clonality.

Table 2.15 Prevalence of virulence factors as detected through PCR.

Gene	Name	Prevalence (%)	Gene	Name	Prevalence (%)
*fnb*A	Fibronectin binding protein A	100	*hlg*	γ-toxin	76
*fnb*B	Fibronectin binding protein B	100	*hlg*-variant	γ-toxin (variant)	80
*clf*A	Clumping factor A	100	*eta*	Exfoliative toxin A	4
*clf*B	Clumping factor B	100	*etb*	Exfoliative toxin B	0
eap	Extracellular adherence protein	100	*tst*	Toxic shock syndrome toxin-1	12
cna	Collagen adhesin	52	*sea*	Staphylococcal enterotoxin A^	80
*sdr*C	Serine-aspartate repeat protein C	44	*seb*	Staphylococcal enterotoxin B	24
*sdr*D	Serine-aspartate repeat protein D	60	*sec*	Staphylococcal enterotoxin C	4
*sdr*E	Serine-aspartate repeat protein E	64	*sed*	Staphylococcal enterotoxin D	0
*ica*A	Biofilm cluster	64	*see*	Staphylococcal enterotoxin E	44
*cap*5	Capsular polysaccharide 5*	52	*seg*	Staphylococcal enterotoxin G	36
*cap*8	Capsular polysaccharide 8*	48	*seh*	Staphylococcal enterotoxin H	0
sak	Staphylokinase^	84	*sei*	Staphylococcal enterotoxin I	48
coa	Coagulase	100	*sej*	Staphylococcal enterotoxin J	8
nuc	Nuclease	100	*sel*	Staphylococcal enterotoxin L	0
chp	Chemotaxis inhibitory protein^	44	*sem*	Staphylococcal enterotoxin M	40
scn	Staphylococcal complement inhibitor^	68	*sen*	Staphylococcal enterotoxin N	44
hla	α-toxin	96	*seo*	Staphylococcal enterotoxin O	40
hlb	ß-toxin^	16	*sep*	Staphylococcal enterotoxin P^	4
hld	δ-toxin	100			

*/^= mutually exclusive

Table 2.16 Associations of virulence factors with methicillin-resistance and clonality. Significant values at a confidence interval of 95% are indicated in bold.

Virulence factor	Association with MSSA/MRSA		Association with clonality	
	MSSA/MRSA	**p-value**	**Clonality**	**p-value**
*fnb*A	MSSA/MRSA	-	All clones	-
*fnb*B	MSSA/MRSA	-	All clones	-
*clf*A	MSSA/MRSA	-	All clones	-
*clf*B	MSSA/MRSA	-	All clones	-
cna	MSSA	0.428	CC15/CC22/CC30	0.113
***sdr*C**	**MRSA**	**0.049**	**CC5/CC8**	**0.030**
*sdr*D	MSSA/MRSA	0.659	CC5/CC22	0.897
***sdr*E**	MSSA	0.397	**CC5/CC8/CC15/CC22**	**0.043**
*ica*A	MSSA	1.000	CC5/CC15/CC22	0.590
eap	MSSA/MRSA	-	All clones	-
***cap*5**	MRSA	1.000	**CC5/CC8/CC22**	**0.005**
***cap*8**	MSSA	0.688	**CC15/CC30**	**0.004**
hlg	MSSA/MRSA	0.615	CC5/CC8/CC15/CC22/CC30	0.400
***hlg*-variant**	MSSA/MRSA	0.615	**CC5/CC8/CC15**	**0.002**
scn	MRSA	1.000	CC5/CC22/CC30	0.799
chp	MSSA	0.414	CC5/CC22/CC30	0.130
hla	MSSA/MRSA	0.400	CC5/CC8/CC15/CC22/CC30	0.640
sak	MSSA/MRSA	1.000	CC5/CC8/CC22/CC30	0.616
coa	MSSA/MRSA	-	All clones	-
nuc	MSSA/MRSA	-	All clones	-
tst	MRSA	0.543	CC30	0.834
sea	MSSA/MRSA	0.615	CC8/CC22/CC30	0.374
seb	MSSA/MRSA	0.653	None	0.731
sec	MSSA	1.000	None	0.240
sed	Not detected	NA	Not detected	NA
see	**MRSA**	**0.049**	CC8/CC30	0.113
seg	MSSA	0.229	**CC5/CC22/CC30**	**0.003**
seh	MSSA	1.000	None	0.640
sei	**MRSA**	**0.015**	**CC5/CC8**	**0.004**

Table 2.16 cont. Associations of virulence factors with methicillin-resistance and clonality. Significant values at a confidence interval of 95% are indicated in bold.

sej	MSSA	0.500	None	0.580
sel	Not detected	NA	Not detected	NA
sem	MSSA	0.211	**CC5/CC22/CC30**	**0.002**
sen	MSSA	0.414	CC5/CC22/CC30	0.052
seo	MSSA	0.211	**CC5/CC22/CC30**	**0.002**
sep	MSSA	1.000	None	0.640
eta	MSSA	1.000	None	0.640
etb	Not detected	NA	Not detected	NA
hlb	MSSA/MRSA	0.626	CC22	0.497
hld	MSSA/MRSA	-	All clones	-

NA = not applicable

2.5 Discussion

2.5.1 High prevalence of PVL:

A prominent finding of this study is the very high prevalence of PVL identified (43.6%), especially among MSSA isolates. This would suggest that the study isolates acquired virulence factor encoding phages before SCC*mec* elements encoding methicillin resistance. The high PVL prevalence in this study is not in concordance with international findings, suggesting that the prevalence of PVL is generally 3-5%[2]. A high PVL prevalence rate has previously only been reported in Thailand/Cambodia[96], where PVL was associated with a better clinical outcome, as well as in some other African countries[169-172]. The PVL prevalence rate among MRSA isolates in this study was 9% vs. 43% in MSSA isolates. This would suggest that there is a significant difference in the PVL prevalence rates of developed vs. developing countries. More studies are required to investigate this further. This data also suggests that a large proportion of this *S. aureus* population consists of PVL+ MSSA isolates from STs that have been associated with MRSA isolates, such as ST22 and ST121[13, 173]. These isolates can thus become a source of new MRSA clones upon the acquisition of a SCC*mec* element. These STs should be monitored. It is also worth noting that all PFGE complexes associated with SSTI infections are all also associated with PVL+ isolates, which indicate either a strong linkage with crucial virulence factors of SSTI infections or a pathogenetic role of PVL during SSTI pathogenesis as suggested by Lina *et al.*[65].

2.5.2 Diversity of MSSA and MRSA clones identified:

In this study, numerous epidemic MRSA clones have been identified, which have all been described in previous international studies[68, 174-176]. The MRSA clones identified in this study include ST22-MRSA-IV (EMRSA-15), ST5-MRSA-I (EMRSA-3), ST239-MRSA-III (Brazilian/Hungarian clone) and ST36-MRSA-II (EMRSA-16), which have all been reported in previous studies from South Africa[119, 120, 123], Africa[170] and worldwide[13]. ST612-MRSA-IV was also identified and was the dominant MRSA clone. This clone has previously only been described in Australia and South Africa[117, 119, 120]. Evidence also emerged which supports the local emergence of this clone (both MRSA and MSSA isolates with the same *spa* types), which has now seemingly adapted to a hospital-based environment in this setting. However, earlier studies conducted focussed on MRSA

only, resulting in a lack of data surrounding MSSA infections. The reason behind the scarcity of CA-MRSA isolates remains unknown.

Numerous endemic MSSA clones have also been described, such as ST15, ST22, ST45, ST121 and ST188[13]. In this study, ST22 was the dominant ST and PFGE clone, THW-C. The isolates of this clone were all predominantly MSSA isolates. ST1862, ST1863, ST1864 and ST1865 were identified as novel STs. These MSSA backgrounds seem to be endemic to this hospital setting and population of *S. aureus*. These novel STs clustered within known CCs, all of which have a known MRSA ST. It is therefore likely that they can serve as lineages for the integration of SCC*mec* elements. ST22 and ST45 are commonly associated with some epidemic MRSA clones[13]. These STs are therefore likely candidates for SCC*mec* acquisition. Several MSSA clones identified here have previously been described from other studies conducted on African isolates[169, 171].

There is an obvious lack of a common genetic background between MSSA and MRSA isolates in this population and we have a very heterogenous population, especially among the MSSA isolates. This either suggests that only some MSSA lineages come into contact with SCC*mec* elements or other bacterial species that can act as possible donors, or only some MSSA lineages can successfully sustain the integration of SCC*mec* elements in the genome.

2.5.3 PFGE as the main typing technique:

Pulsed-field gel electrophoresis was an excellent technique to use to establish clonal relatedness and dominance in the investigated setting and showed the highest DI (displayed in Table 2.12) and supplementing the PFGE data with data generated through *spa*, *agr* and SCC*mec* typing enhanced our ability to elucidate the population structure of *S. aureus*. Isolates of the ST239-MRSA-III-t037 (CC8) background and of the CC30 background were clustered in a single CC by BURP, *spa*-CC021. This is due to the chromosomal integration of a large chromosomal fragment from ST30 in ST8, leading to the evolution of ST239[63].

PFGE, supplemented with *spa* typing and MLST has previously been used to successfully investigate the population structure of *S. aureus*[140, 168, 177]. Our data suggest that using both techniques in combination yields a better resolution that a single one. Sequence-based typing based on regions of the *clfB* and *fnb* genes have also been described and may be worth investigating[178] as supplementary techniques to PFGE.

2.5.4 Population structure of *S. aureus* at Tygerberg hospital:

Using molecular information combined with an analysis of clinical category of isolates with strain types and strengthening it through statistical associations with clinical categories, we were able to effectively describe the population structure of *S. aureus* at Tygerberg hospital over a one year period.

Our investigation of the population structure revealed the presence of numerous clones. Some clones were exclusively MSSA, while others were exclusively MRSA and some were mixed. The clones will be discussed in more detail from an MLST perspective, as this makes comparisons with other studies easier. MSSA clones will be named according to MLST-methicillin-resistance-PFGE, and MRSA clones according to MLST-methicillin resistance-SCC*mec* type-PFGE. Mixed clones will initially be referred to by MLST-PFGE only.

The dominant clone identified in this population was ST22-THW C (major) and could be sub-divided based on *spa* typing, as a sub-founder t022 was also identified within *spa*-CC891 using the BURP algorithm. ST22-MSSA-THW C contained 59 PVL+ isolates (92%) and was predominantly *spa* t891. One PVL+ MRSA isolate, ST22-MRSA-V (*spa* t891) was also identified and has previously been reported in Australia[23]. The presence of this MRSA isolate indicates that a SCC*mec* acquisition event has occurred not too long ago. This MRSA clone could be the first of a CA-MRSA lineage that may potentially be associated with SSTI as ST22-MSSA-THW C was found to be statistically associated with SSTI infections caused by PVL+ MSSA isolates. ST22-MRSA-V isolates have been identified in India[179] and other ST22-MRSA isolates, such as the Barnim clone, in Germany[180]. Four other isolates were also identified to possess *spa* types closely related to *spa* t022. These isolates were all PVL- MRSA isolates and contained SCC*mec* type IV. ST22-MRSA-IV is usually associated with the epidemic MRSA clone EMRSA-15, which was a dominant clone in the UK for decades[13, 23, 181], Spain[181, 182] since 2004, in non-hospitalised patients in Australia since 2002[183] and in Irish hospitals since 2002[184]. It is still one of the dominant clones in India[179], Spain[181] and the Atlantic Azores Islands[185] today. It is worth noting that ST22 is a known epidemic MRSA lineage, usually associated with SCC*mec* IV, ST22-MRSA-IV/EMRSA-15[186]. Yet here in this population it is established as the dominant MSSA clone, with one MRSA isolate with a SCC*mec* V element. Only four SCC*mec* IV containing isolates were identified. The data might thus suggest that this lineage may likely give rise to a CA-MRSA clone (possibly ST22-MRSA-IV or ST22-MRSA-V), which can become epidemic.

The second dominant clonal complex was CC30. It consisted out of ST1865-MSSA-THW A (major) and ST36-MRSA-II-THW B (intermediate). Both clones were classified as *spa*-CC021. Strains from ST1865-MSSA-THW A were predominantly PVL+. This clone was statistically also associated with SSTI infections caused by PVL+ MSSA isolates (see Table 2.13 and Table 2.14). ST1865 was identified as a novel MLST ST during this study. ST36-MRSA-II-THW B was associated with infections in adults caused by PVL- MRSA isolates. ST36-MRSA-II is normally associated with the epidemic clone EMRSA-16[23, 186] or USA200[68] and is found in Irish hospitals[184, 187], the Canary Islands[188], Canada[189] and Spain[182]. ST1865 can act as a possible recipient for SCC*mec* elements.

CC8 is the 3rd dominant clonal complex and ST8, ST612, ST239 and ST1862 were all identified as part of this clonal complex. Two MRSA STs were identified. ST612, a double locus variant (dlv) of ST8, was the only MLST ST subdivided by PFGE into two clones, THW-O (major) and THW-N (intermediate). ST612-MRSA-IV-THW O was the dominant MRSA clonal complex identified in this population and was associated with PVL- MRSA isolates. This was also the case for THW-N. One MSSA isolate was present in THW-O and had the same *spa* type as a MRSA isolate, t1443. This can possibly represent the local acquisition of SCC*mec* by MSSA isolates, leading to the rise of a local MRSA clone. As only one MSSA isolate was identified, it can be seen that the MSSA members have largely been replaced by their MRSA counterparts. ST612-MRSA-IV has previously been described as the dominant MRSA clone in Cape Town[120], South Africa[119] and Australia[117] and seems to have adapted to a hospital environment. ST612 is a double-locus variant (dlv) of ST8. ST239-MRSA-III-THW L was also identified and associated with CC8 and is probably the oldest pandemic MRSA clone[23]. This clone has been epidemic on virtually every continent, and is prevalent in South America[174, 190], Europe[13], India[179], Finland[191], China[192-194], Australia[183], Malaysia[195], Turkey[196], India[197] and various Asian countries[198], including Taiwan[199]. ST1862-MSSA-THW G (intermediate) was associated with MSSA SSTI infections in adults. ST1862 was identified as a novel MLST ST. The final member of this CC was ST8-MSSA-THW J (intermediate). ST8-MRSA can be associated with both HA-MRSA (USA500) and CA-MRSA (USA300) isolates[13]. In this study, this clone was associated with infections in adults caused by PVL- isolates. All members of this clone carried *spa* t1476 and two MRSA isolates were also present, carrying SCC*mec* type V. This data, again, supports the local acquisition of SCC*mec* elements and these ST8-MRSA-V isolates might be the first of a new CA-MRSA clone. CC8-MRSA-V CA-MRSA isolates have previously been reported in Australia[183] and Germany[23]. Other CA-MRSA ST8-MRSA isolates have been reported in the UK[200], Germany[201] and Belgium[202].

Three members of CC15 were identified: ST1-MSSA-THW V2 (major), associated with infections caused by MSSA isolates; ST15-MSSA-THW S (major), associated with infections caused by PVL-MSSA isolates; and ST188-MSSA-THW X (intermediate), also associated with infections caused by PVL- MSSA isolates. ST15 is known worldwide to be associated with MSSA isolates and that MRSA isolates from this genetic background are scarce[13], although "early" MRSA isolates have been identified from this ST[203]. ST1-MRSA-IV is associated with the USA400 clone[13, 68], a CA-MRSA clone prevalent in the USA[68], Korea[204], Australia[183], USA[181], UK[205] and China[206].

ST45-MSSA-THW EE (major) was identified in a PFGE complex of 37 isolates, was characterised as a singleton by MLST, and was associated with PVL- MSSA isolates. This ST is commonly associated with an epidemic MRSA clone, the Berlin clone[23], which has been identified in the UK and the Netherlands[13, 23], and is also referred to as the USA600 (ST45-MRSA-IV) clone[68]. This ST has been identified in German MSSA and MRSA isolates[180, 207], as well as in isolates from patients in nursing homes in Belgium[202]. ST45-MRSA-I has also been identified from marine water and intertidal beach sand from public beaches[208] in western Washington state in the USA. This ST can be a very likely recipient of a SCC*mec* element.

CC5 was composed out of three STs: ST5-MRSA-I-THW AA (intermediate), associated with infections caused by PVL- MRSA isolates; ST1863-MSSA-THW Y (major) and ST1864-MSSA-THW Z (intermediate), both associated with infections caused by PVL- MSSA isolates. ST1863 and ST1864 were identified as novel MLST STs during this study and are both single-locus variants (slvs) of ST5. ST5-MRSA-I has previously been associated with the EMRSA-3 clone[13] and has been identified in strains from Australia[23], Germany[23] and Argentina[209]. ST5-MRSA-IV is associated with the paediatric clone[13]. ST5 isolates, or variants thereof, have been associated with reduced susceptibilities to vancomycin[210], especially isolates carrying the SCC*mec* type II elements. Two MSSA lineages exist which are closely related to ST5 and are endemic to this setting. It is possible that this ST5 emerged locally after it underwent evolutionary changes after acquisition of a SCC*mec* I element. ST5 has also recently been described as the dominant ST in Gabon, Central Africa[211].

ST121-MSSA-THW E (major) was associated with SSTI caused by PVL+ MSSA isolates. This ST is commonly only associated with a MSSA background, and has previously been reported[23, 212]. This ST is a common, global cause of SSTI[213]. Although MRSA isolates have been described from this ST, they are usually rare[23]. This ST has also been associated with *S. aureus* infection in cattle[214].

ST6 (major) was associated with PVL- MSSA isolates. This ST is an endemic MSSA lineage.

ST12 (intermediate) was associated with PVL+ MSSA isolates. MRSA isolates of this ST have been identified in Ireland, Norway and Australia, and most of them seem to be PVL-[23]. This ST is usually only associated with MSSA strains[13]. ST12-MRSA-IV isolates have been identified in Ireland[184].

ST97 (intermediate) was associated with infections in males caused by PVL- MSSA isolates. This ST can commonly be associated with *S. aureus*-based disease in cattle[23, 214]. MRSA isolates from this ST are quite rare.

ST88 (intermediate) was associated with SSTI caused by PVL+ isolates and is also commonly associated with CA-MRSA, which has been reported in the UK, Abu Dhabi, Spain and Nigeria[23], as well as among Chinese paediatric patients[206, 215]. This ST can also be a potential CA-MRSA clone in our setting, as it is known that PVL+ SSTIs are commonly associated with CA-MRSA. However, to date, CA-MRSA in our setting and in South Africa in general are scarce.

2.5.5 Local SCC*mec* evolution and acquisition:

Three MRSA clones were also identified which have possibly emerged locally. Population structure data supports the local acquisition of type V SCC*mec* elements by two clones, leading to the emergence of ST22-MRSA-V and ST8-MRSA-V. These clones are very likely CA-MRSA clones and can potentially lead to epidemics. These strains are fortunately not present in abundance. The 3rd clone which could have emerged locally is the dominant MRSA clone, ST612-MRSA-IV. Only one MSSA isolate was identified in this CC, so it is likely that this clone acquired the SCC*mec* type IV element some time ago.

Also, one isolate from ST239-MRSA-THW J possessed a non-typeable SCC*mec* element, while the remaining isolates possessed SCC*mec* III elements. This data suggests that replacement of a SCC*mec* element by another has taken place in this population, resulting in the generation of new and different MRSA clones.

2.5.6 Statistical association with HIV and other virulence factors:

Two clones were identified to be statistically associated with an HIV+ status: ST8-MSSA-THW K (minor) and ST612-MRSA-IV-THW P (minor). Both clones were classified as MLST CC8, which has been reported previously[132, 134, 136]. ST8 isolates have also been reported as USA300 isolates and have been associated with necrotising disease in HIV+ persons[134]. ST612 has been described as the dominant ST, however from a different PFGE clone. The statistical association with patient HIV status could not be determined due to the high number of patients with an unknown HIV status. Further studies on the association between bacterial clonality and a HIV+ status are required.

Regarding the virulence factor investigations, it can be noted that all isolates possessed genes involved in adherence (*fnb*A/B, *clf*A/B, *eap*) and invasion (*fnb*A/B, *eap*) of host cells. Diep *et al.* identified a similar pattern in their collection of HA- and CA-MRSA isolates[216]. Also, genes predominantly involved in the induction of cell death (*hla*), evasion of the host immune system and toxins with superantigenic properties were present in most isolates. Some toxin genes were only moderately present or even completely absent. All isolates possessed either *cap*5 or *cap*8, but never both, as has been reported previously[85, 216]. Eighty percent of the isolates possessed the known IEC of *scn*, *chp*, *sak* and *sea* or *sep*, or a combination of these genes, while 25% possessed all five genes. This correlated very well with the low prevalence of *hlb*, since this IEC are carried on phages which integrate into the *hlb* gene[86]. Again the data indicates the on-going interaction between local *S. aureus* strains and various phages. The genes *seg*, *sei*, *sem*, *sen* and *seo*, also known as the enterotoxin gene cluster, *egc*, were detected in most isolates and in combinations. The *egc* was completely absent from all CC15 isolates (ST15, ST1 and ST188) as well as ST12, ST6 and ST8 isolates. All five genes were detected in CC5 isolates only. There seem to be an association between the genes present in the *egc* and clonality, as has been identified before[216, 217]. Unlike previously published by Deurenberg *et al.*, *cna* and *tst* could not be used to distinguish CC15 and CC30 isolates[218]. In general, we see variation in the association of certain virulence factors with clonality or methicillin-resistance, which probably does not influence pathogenesis, as previous studies have shown[219].

The expression of core-genome virulence components, such as *hla* and *psmα*, has been linked to the increased virulence of some CA-MRSA lineages, such as the USA300 complex[50]. The gene *hla*

was identified in 24/25 representative isolates. Further analyses will reveal which of the isolates could be classified as being cytotoxic.

2.6 Conclusion

The population studied here is composed out of an extremely diverse population of endemic MSSA clones and epidemic MRSA clones, and an absence of a common genetic background between MSSA and MRSA isolates has been identified. The molecular typing techniques used in combination were sufficient to elucidate and study the population structure of *S. aureus*, allowing us to identify various clones, some of which were associated with certain clinical categories.

We were also able to indicate that three of the MRSA clones identified emerged locally through the acquisition of SCC*mec* elements, including the unique, dominant MRSA clone, ST612. Few of the MSSA lineages offer a stable enough genetic background for the maintained integration of SCC*mec* elements, leading to the rise of different MRSA clones.

A prominent finding of this study is the high PVL prevalence identified. However, CA-MRSA and PVL+ MRSA isolates are scarce in this population. The MSSA population identified and studied can act as perfect reservoirs for potential CA-MRSA clones upon the acquisition of SCC*mec* elements, leading to the rise of PVL+ CA-MRSA clones.

All the representative isolates selected possessed the genotypic means for the effective adherence to cellular matrices, invasion of host cells and induction of host cell death. Genes associated with host immune evasion were also present in high numbers.

In the following chapters host-pathogen interaction characteristics of the selected representative isolates will be investigated by focusing on their abilities to adhere to selected ligands, invade host cells and also induce the death of these cells.

2.7 Appendix A

Table A1 displays the results obtained for all representative isolates for the genotypic PCR investigation into the prevalence of the selected virulence factors.

Table A1 Genotypic PCR results obtained of the prevalence of the selected virulence factors in the representative isolates.

Isolate name	PFGE	MLST		Invasion			Adherence						Persistence	Other enzymes		
		ST	CC	*fnb*A	*fnb*B	*eap*	*clf*A	*clf*B	*cna*	*sdr*C	*sdr*D	*sdr*E	*ica*A	*sak*	*coa*	*nuc*
THW38	THW-A	1865	30	+	+	+	+	+	+	-	-	-	-	+	+	+
THW382	THW-B	36	30	+	+	+	+	+	+	-	+	-	+	+	+	+
THW146	THW-C	22	22	+	+	+	+	+	+	-	+	+	+	+	+	+
THW393	THW-C	22	22	+	+	+	+	+	+	-	+	+	+	+	+	+
THW262	THW-O	612	8	+	+	+	+	+	-	+	+	+	-	+	+	+
THW70	THW-N	612	8	+	+	+	+	+	+	+	+	+	+	+	+	+
THW65	THW-G	1862	8	+	+	+	+	+	-	-	+	+	-	+	+	+
THW271	THW-J	8	8	+	+	+	+	+	+	-	-	-	-	+	+	+
THW81	THW-L	239	8	+	+	+	+	+	-	+	-	-	+	+	+	+
THW195	THW-K	8	8	+	+	+	+	+	-	+	-	+	+	+	+	+
THW273	THW-P	612	8	+	+	+	+	+	-	+	+	+	+	-	+	+
THW93	THW-O	612	8	+	+	+	+	+	-	+	-	+	+	+	+	+
THW99	THW-L	239	8	+	+	+	+	+	-	+	-	-	-	+	+	+
THW122	THW-S	15	15	+	+	+	+	+	+	-	+	+	-	-	+	+
THW412	THW-V2	1	15	+	+	+	+	+	+	-	-	+	+	-	+	+
THW224	THX-X	188	15	+	+	+	+	+	-	+	-	+	+	+	+	+
THW356	THW-EE	45	45	+	+	+	+	+	+	-	+	-	-	+	+	+
THW17	THW-Y	1863	5	+	+	+	+	+	+	+	+	+	+	+	+	+
THW235	THW-Z	1864	5	+	+	+	+	+	-	+	-	+	+	+	+	+
THW264	THW-AA	5	5	+	+	+	+	+	-	+	+	+	+	-	+	+
THW64	THW-V1	6	6	+	+	+	+	+	-	-	+	+	-	+	+	+
THW366	THW-E	121	121	+	+	+	+	+	+	-	-	-	+	+	+	+
THW368	THW-V3	12	12	+	+	+	+	+	+	-	+	-	+	+	+	+
THW241	THW-W	97	97	+	+	+	+	+	-	-	+	+	+	+	+	+
THW255	THW-BB	88	88	+	+	+	+	+	+	-	+	-	-	+	+	+

Table A1 cont. Genotypic PCR results obtained of the prevalence of the selected virulence factors in the representative isolates.

Isolate name	PFGE	MLST		Immune evasion				Hemolysins					Exfoliative Toxins	
		ST	CC	*cap*5	*cap*8	*scn*	*chp*	*hla*	*hlb*	*hld*	*hlg*	*hlg*v	*eta*	*etb*
THW38	THW-A	1865	30	-	+	+	+	+	-	+	+	-	-	-
THW382	THW-B	36	30	-	+	+	+	+	-	+	+	-	-	-
THW146	THW-C	22	22	+	-	+	+	+	-	+	+	-	-	-
THW393	THW-C	22	22	+	-	+	+	+	+	+	+	-	-	-
THW262	THW-O	612	8	+	-	-	-	+	-	+	+	+	-	-
THW70	THW-N	612	8	+	-	+	-	+	-	+	+	+	-	-
THW65	THW-G	1862	8	+	-	-	-	+	-	+	-	+	-	-
THW271	THW-J	8	8	+	-	-	+	+	-	+	-	+	-	-
THW81	THW-L	239	8	-	+	+	-	+	-	+	+	+	-	-
THW195	THW-K	8	8	+	-	-	+	+	+	+	-	+	-	-
THW273	THW-P	612	8	+	-	+	-	+	-	+	+	+	-	-
THW93	THW-O	612	8	+	-	+	-	+	-	+	+	+	-	-
THW99	THW-L	239	8	-	+	+	-	+	-	+	+	+	-	-
THW122	THW-S	15	15	-	+	-	+	+	-	+	+	+	+	-
THW412	THW-V2	1	15	-	+	-	-	+	+	+	+	+	-	-
THW224	THX-X	188	15	-	+	+	-	+	-	+	+	+	-	-
THW356	THW-EE	45	45	-	+	-	+	+	-	+	-	-	-	-
THW17	THW-Y	1863	5	+	-	+	+	+	-	+	+	+	-	-
THW235	THW-Z	1864	5	+	-	+	+	+	-	+	+	+	-	-
THW264	THW-AA	5	5	+	-	-	-	-	-	+	+	+	-	-
THW64	THW-V1	6	6	+	+	+	-	+	-	+	-	+	-	-
THW366	THW-E	121	121	-	+	+	-	+	-	+	+	+	-	-
THW368	THW-V3	12	12	-	+	+	-	+	-	+	+	+	-	-
THW241	THW-W	97	97	+	-	+	-	+	-	+	+	+	-	-
THW255	THW-BB	88	88	-	+	+	+	+	+	+	+	+	-	-

Table A1 cont. Genotypic PCR results obtained of the prevalence of the selected virulence factors in the representative isolates.

Isolate name	PFGE	MLST		Superantigens and toxin-mediated disease														
		ST	CC	*tst*	*sea*	*seb*	*sec*	*sed*	*see*	*seg*	*seh*	*sei*	*sej*	*sel*	*sem*	*sen*	*seo*	*sep*
THW38	THW-A	1865	30	-	+	-	-	-	+	+	-	-	-	-	+	+	+	-
THW382	THW-B	36	30	+	+	-	-	-	+	+	-	-	-	-	+	+	+	-
THW146	THW-C	22	22	-	+	-	-	-	-	+	-	-	-	-	+	+	+	-
THW393	THW-C	22	22	-	+	-	-	-	-	+	-	-	-	-	+	-	+	-
THW262	THW-O	612	8	-	+	+	-	-	+	-	-	+	-	-	-	-	-	-
THW70	THW-N	612	8	-	+	-	-	-	+	-	-	+	-	-	-	-	-	-
THW65	THW-G	1862	8	-	-	-	-	-	-	+	-	-	-	-	+	+	+	-
THW271	THW-J	8	8	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-
THW81	THW-L	239	8	-	+	-	-	-	+	-	-	+	-	-	-	-	-	-
THW195	THW-K	8	8	+	+	+	-	-	-	-	-	+	+	-	-	+	-	-
THW273	THW-P	612	8	-	+	+	-	-	+	-	-	+	-	-	-	+	-	-
THW93	THW-O	612	8	-	+	+	-	-	+	-	-	+	-	-	-	-	-	-
THW99	THW-L	239	8	-	+	-	-	-	+	-	-	+	-	-	-	-	-	-
THW122	THW-S	15	15	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
THW412	THW-V2	1	15	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
THW224	THX-X	188	15	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
THW356	THW-EE	45	45	-	+	-	+	-	-	-	-	-	+	-	+	+	+	-
THW17	THW-Y	1863	5	-	-	-	-	-	+	+	-	+	-	-	+	+	+	+
THW235	THW-Z	1864	5	-	+	+	-	-	-	+	-	+	-	-	+	+	+	-
THW264	THW-AA	5	5	-	-	-	-	-	-	+	-	+	-	-	+	+	+	-
THW64	THW-V1	6	6	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-
THW366	THW-E	121	121	-	+	+	-	-	-	+	-	-	-	-	+	+	+	-
THW368	THW-V3	12	12	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-
THW241	THW-W	97	97	-	+	-	-	-	-	-	-	+	-	-	-	-	-	-
THW255	THW-BB	88	88	-	+	-	-	-	-	-	-	+	-	-	-	-	-	-

CHAPTER 3: Adherence of representative clinical *S. aureus* isolates to specific immobilised human ligands

3.1 Introduction

3.1.1 Host extracellular matrix (ECM) components involved in bacterial adherence:

Fibronectin, fibrinogen and collagen are three of many different extracellular matrix (ECM) molecules found in macromolecular structures such as the skin, cartilage or epithelial lining. Both fibronectin and fibrinogen play significant roles in the binding of *S. aureus* to the skin during infections associated with skin diseases such as atopic dermatitis[220]. Fibronectin is a component of ECM[221], human plasma and connective tissue[222] and aggregates Gram-positive bacteria[221]. Mechanical stretching of this protein promotes disruption of bacterial adherence[223] by physically destroying epitopes. Numerous human serum proteins can also act as ligands for *S. aureus* adherence[224]. Fibrinogen-binding is commonly associated with infective endocarditis[225], while collagen is commonly required for the colonisation of cartilage[226].

3.1.2 Staphylococcal adherence proteins:

Numerous bacterial surface proteins can be utilised during the process of adherence to host ligands. These proteins are called “microbial surface components recognising adhesive matrix molecules”, or MSCRAMM, such as fibronectin-binding proteins A and B[3], Staphylococcal Protein A and Clumping factors and are anchored to the bacterial cell wall through a LPXTG motif[100]. The organism can establish an infection through the expression of these cell wall-anchored proteins during the logarithmic phase of growth, aiding in the infection of host tissue. Another group of bacterial proteins which is involved in this process are the SERAM molecules, or the “secreted

expanded repertoire adhesive molecules"[102], such as the extracellular adherence protein (Eap), commonly found in supernatant and secreted during the logarithmic phase.

3.1.2.1 Protein A (SpA):

The exact function of Staphylococcal Protein A remains unclear, but it is known that this protein can bind the Fc domain of eukaryotic immunoglobulin G (IgG)[147, 148] and inhibit opsono-phagocytosis[100, 148]. This surface protein also binds strongly to the Fab region V_H3 heavy chain-bearing immunoglobulins (Ig)[227] and to von Willebrand factor (vWF)[227] and has also been implicated in the adherence to mesothelial cell monolayers[228]. This protein also binds directly to osteoblasts, resulting in the prevention of their proliferation and the induction of apoptosis, characteristic for a debilitating disease such as osteomyelitis[229]. Please refer to Figure 3.1 for a structural comparison of this protein to other *S. aureus* surface proteins.

3.1.2.2 Fibronectin-binding proteins (FnbPs):

Adherence to fibronectin by *S. aureus* can be mediated by fibronectin-binding proteins A and B (FnBPA/B) and also aids in the binding of the organism to plasma clots[100, 230]. These proteins are present in virtually every clinical *S. aureus* isolate. Both genes are fundamental for the invasion of eukaryotic cells[102]. The A domain of FnbA has a fibrinogen-binding function[231] and adheres to and activates human platelets[83, 232]. Sinha *et al*. demonstrated that FnBPs are sufficient for the invasion of eukaryotic host cells[104]. Adherence to fibronectin by FnbA is mediated by multiple, substituting binding regions present on the protein[233], but this protein has to be anchored in the bacterial cell wall for it to be fully functional[89], i.e. adhere to and invade mammalian cells. A novel biofilm phenotype, stimulated by FnBPA and FnBPB has also been identified, and occurs independently from known ligand binding activities of either protein[114].

FnbA can also mediate human T lymphocyte adhesion and co-activation[234] Please refer to Figure 3.1 for a structural comparison of this protein to other *S. aureus* surface proteins.

3.1.2.3 SD repeat proteins (Sdr):

These proteins are structurally characterised by the presence of a Serine-Aspartate (Ser-Asp) dipeptide rich repeat region called the R domain[100]. ClfA/B are members of this family of proteins and three other members, SdrC/D/E have also been identified. The exact roles of these proteins are still poorly understood. A *sdr*C^{+}*sdr*D^{-}*sdr*E^{-} phenotype has previously been associated with MSSA strains[235], while the presence of *sdr*D was strongly associated with MRSA strains[235].

S. aureus possess two distinct fibrinogen-binding proteins, namely clumping-factor A and B (ClfA/B), of which ClfA is mainly used to adhere to substances containing fibrinogen or to clump in its presence[100, 236]. ClfA has been implicated in infective endocarditis[83, 225] and other endovascular diseases[237] and inhibits phagocytosis in the absence of fibrinogen[238] (the exact mechanism remains unknown), while ClfB plays a role during colonisation through mediating adherence to epithelial cells[239, 240]. Please refer to Figure 3.1 for a structural comparison of this protein to other *S. aureus* surface proteins.

3.1.2.4 Collagen adhesin (Cna):

This protein is mainly responsible for bacterial adherence to collagen substrates and collagenous tissue[100, 241-243] as eight ligand-binding sites are present on the protein[244, 245] and is present in only 30-60% of clinical isolates[100]. This protein is also required for adherence to human cartilage[246] and has been shown to bind human collagen I - VI[226]. This protein is a virulence determinant for *S. aureus*-based experimental endocarditis[247] and keratitis[248]. Please refer to Figure 3.1 for a structural comparison of this protein to other *S. aureus* surface proteins.

3.1.2.5 Extracellular adherence protein (Eap):

This multifunctional protein is known to have a broad specificity for host proteins[249], recognising fibronectin, fibrinogen and collagens[83, 250] *in vitro*. It is required as a full-length protein to act as an invasin for *S. aureus*[88, 251]. It is present in >96% of all clinical isolates in various sizes and

lacks the typical LPXTG domain, although Hussain *et al.* identified it in all their isolates screened[165]. Anti-inflammatory[252] and immune-modulatory roles[253, 254] have been described for this protein[83, 251]. It is structurally related to *S. aureus* superantigens, but does not act as a superantigen[255], unlike some of the classical *S. aureus* superantigens (e.g. TSST-1, SEA, SEB etc.).

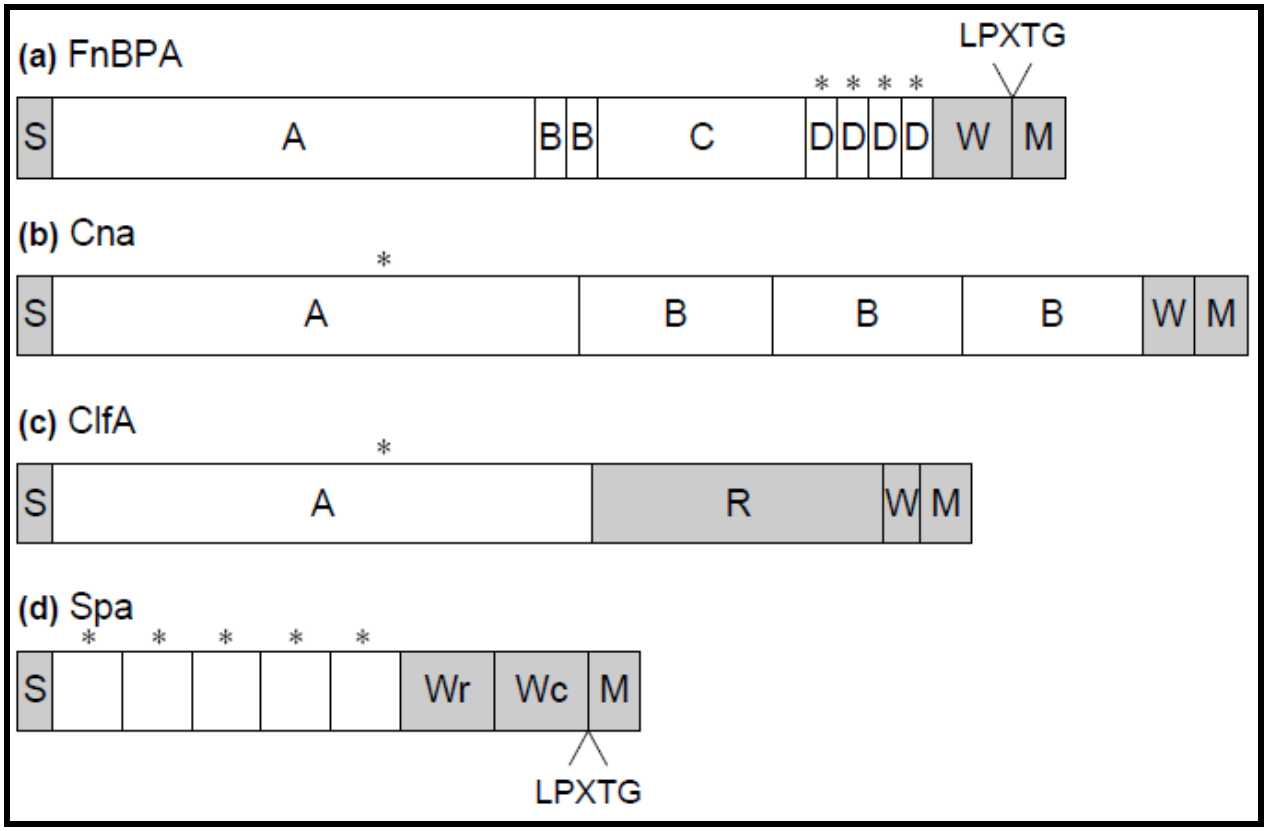


Figure 3.1 The structural organisation of the *S. aureus* MSCRAMM proteins (a) fibronectin-binding protein A; (b) collagen-binding protein; (c) clumping factor A; and (d) protein A. S = signal sequence; W = wall-spanning region; M = membrane-spanning region and positively charged residues; R = Ser-Asp dipeptide region; Wr = octapeptide repeat region; Wc = non-repeated region; * = ligand-binding domains; A-D= domains; LPXTG = cell-wall anchor domain. Adapted from Foster *et al.*[100].

3.1.2.6 Plasmin-sensitive protein (Pls):

This protein is a member of the Sas (*Staphylococcus aureus* surface proteins) family and has until now only been identified in MRSA isolates carrying the SCC*mec* I element[83]. This protein acts by steric hindrance, resulting in decreased adherence to host cells and decreased invasiveness of host cells[256, 257] and experimental data has identified it as a virulence factor involved in septic arthritis[258]. Cellular invasiveness can also be predicted by the SCC*mec* and *spa* type, in addition to the expression of *pls*[259].

3.2 Aims and Objectives

3.2.1 Study aim:

The aim of the research presented in this chapter was to investigate the abilities of the representative *S. aureus* isolates selected from different clonal complexes, to adhere to various immobilised ligands and identify any potential associations between adherence and bacterial characteristics, such as clonality. The same representative isolates as in the previous chapter were used.

3.2.2 Study objectives:

1. To establish a baseline of adherence by using non-coated plastic plates.
2. To investigate the abilities of the representative isolates to adhere to plates coated with human plasma proteins and human serum proteins.
3. To investigate the abilities of the representative isolates to adhere to plates coated with selected human ligands:
    a. Fibronectin (prominent plasma/serum component; prominent matrix component);
    b. Fibrinogen (prominent plasma component);
    c. Collagen IV (important accessible matrix component);
    d. Collagen VI (important accessible matrix component).
4. To investigate if any statistical associations are present between adherence and:
    a. Clonality;
    b. Methicillin-resistance;
    c. Bacterial PVL status, i.e. PVL+ or PVL-; and
    d. Patient HIV status.

3.3 Materials and Methods

3.3.1 Chemicals, reagents and media:

All chemicals, reagents and consumables used during this research were analytical and/or molecular biology grade. Human plasma fibronectin was obtained from Millipore (Billerica, USA), while human plasma fibrinogen was obtained from Merck Chemicals (Darmstadt, Germany). Human collagen types IV and VI were obtained from Rockland Inc. (Gilbertsville, USA). Only human derived ligands were used during this study.

3.3.2 Bacterial strains and growth conditions:

All bacterial isolates used were sub-cultured on 5% Blood agar plates (Bio-Merieux, France) after collection and incubated overnight at 37 ºC. The same 25 isolates selected in Chapter 2 were used as representative isolates. Please refer to Table B1 (Appendix B). 2-3 colonies of bacteria were used to inoculate 20 ml Mueller-Hinton broth (MHB), which was incubated statically for 16 h at 37 °C. The reference isolate NCTC8325-4 was used as a positive control for all adherence assays.

3.3.3 Preparation of human plasma and serum:

Human plasma and human serum proteins, required to coat the clear polystyrene 96-well flat-bottomed plates (Sarstedt, Germany), were obtained from the blood of a healthy male volunteer donor and a healthy female volunteer donor. Blood was collected in a serum tube for serum proteins and an EDTA-containing plasma tube for plasma proteins. The serum tubes were left at room temperature (RT = 25 ℃) for 3 h after blood collection to allow for clot formation. All blood collection tubes were centrifuged at 2 800 rpm for 30 min, where after the plasma and serum were transferred to new, sterile 50ml tubes, which were centrifuged at 1 300rpm for 10 min. The plasma and serum was again transferred to new, sterile 50 ml tubes. All plasma and serum proteins were heat inactivated in a water bath at 56 °C and stored at -20 °C as 1ml aliquots.

3.3.4 Coating of 96-well micro-titre plates with human-derived ligands:

96-well plates (Sarstedt, Germany) were coated with a specific ligand using a modified method of Peacock *et al*[163]. Briefly, the specific ligand was diluted in 1× PBS to a concentration of 10 µg/ml, of which 100 µl was added to every well of a clear, sterile, 96-well flat-bottomed plate (Sarstedt, Germany), from now on referred to as the plate. The plate was incubated at 37 °C for 1 h and washed once with 200 ul 1× PBS per well, using a pump aspirator. 200 µl of 2% human serum albumin (HSA) in 1× PBS was added to every well to block any non-specific sites in the wells and left at 4 °C o/n, where after the plate was washed ×3 with 200 µl 1× PBS per well. The plate was left to air dry for 5 min in a sterile laminar flow hood before bacterial inoculation. A plate was coated freshly every time an adherence assay was set-up.

3.3.5 Adherence assay:

All adherence assays were performed with standardised bacterial cultures of $OD1_{600nm}$ to allow for direct comparisons between isolates. All isolates and controls were tested in triplicate wells and three independent experiments were performed.

Bacterial cultures were prepared as described in section 2.3.2. After incubation, the cultures were centrifuged at 4 000rpm for 5 min and the supernatant discarded. The bacterial pellet was washed once with 1× PBS (complete resuspension of the pellet by vortexing) and centrifuged again (4 000rpm, 5 min). The supernatant was discarded and the bacterial pellet was completely resuspended in 5 ml 1% HSA/PBS by vortexing. Following this, the bacterial OD was measured and adjusted to OD 1_{600nm} as follows. Briefly, a 1:10 dilution in 1% HSA/PBS of each bacterial culture was prepared inside a cuvette and measured at 600nm. The amount of bacterial culture required for 1ml of adjusted culture at OD 1 was calculated and the bacterial culture was adjusted. After adjustment, a 1:10 dilution in 1% HSA/PBS was prepared of the adjusted culture and measured at 600nm. Only adjusted cultures between OD 0.95 - 1.05 were accepted. Adjusted cultures above or below these limits were adjusted for a second time and measured.

20 µl of the OD 1 bacterial culture was used to inoculate 200 µl MHB in a 1.5 ml tube and vortexed, of which 200 µl was used to inoculate a well of a freshly coated plate. After inoculation, the plate was closed and statically incubated at 37 °C with 5% CO_2 o/n. All representative isolates

were tested in triplicate wells. As previously stated, strain NCTC8325-4 was used as a positive control isolate. Two negative controls were included: 200 µl 1× PBS without bacterial inoculum and 200 µl MHB without bacterial inoculum.

After incubation, the medium was exhausted using an aspirator pump and each well was washed once with 200 µl 1× PBS. After the 1× PBS was removed, the plate was allowed to air dry for 5min inside a sterile laminar flow hood. 100 µl 0.1% crystal violet solution was added to each well and incubated at RT for 30 min. The crystal violet solution was exhausted and every well was washed ×3 with 200 µl 1× PBS. The plate was allowed to air dry under a sterile laminar hood for 5 min, where after 200 µl elution buffer was added to every well and incubated at RT o/n to allow for the elution of the crystal violet from the adherent bacteria.

The following day the elution buffer was measured using an ELISA reader, the TECAN Infinite Pro 200 (Tecan GmbH, Germany) at 620nm.

Adherence to a specific ligand was expressed as a percentage relative to the positive control, which was set at 100%, after subtraction of the PBS negative control from every reading. The standard error of mean (SEM) and the standard deviation (SD) of every mean was determined.

Adherence of the representative isolates was tested first in uncoated plates, to establish a baseline of adherence (presence of no ligand).

A single 96-well plate included all the representative isolates (n = 25) and the three controls (1× positive control (NCTC8325-4) and 2× negative controls (1× PBS, MHB) in triplicate. Three independent experiments were performed.

3.3.6 Statistical associations:

3.3.6.1 Associations between adherence and methicillin-resistance or clonality:

To investigate if any associations existed between adherence and methicillin-resistance, two tests were performed. Firstly, as a parametric comparison, the 2-sample t-test was also used. A confidence interval of 95% was used and an association was regarded as statistically significant if a p-value < 0.05 was obtained. Secondly, as a non-parametric comparison, the 2-sample Wilcoxon rank-sum test (Mann-Whitney test) was used. An association was regarded as statistically significant if a p-value < 0.05 was obtained.

To investigate if any associations existed between adherence and clonality, the Kruskal-Wallis test was performed as a comparison using non-parametric inference. In order for us to compare all groups in a valid fashion, a group was created which was composed of all the singletons. An association was regarded as statistically significant if a p-value < 0.05 was obtained.

3.3.6.2 Associations between adherence and patient HIV status or bacterial PVL status:

To investigate if any associations existed between adherence and patient HIV status, the Kruskal-Wallis test was performed as a comparison using non-parametric inference. An association was regarded as statistically significant if a p-value < 0.05 was obtained.

To investigate if any associations existed between adherence and bacterial PVL status, i.e. PVL-positive or negative, the 2-sample Wilcoxon rank-sum test (Mann-Whitney test) was used. An association was regarded as statistically significant if a p-value < 0.05 was obtained.

For all statistical investigations, the software package Stat v.12 was used.

3.3.7 Composition of media, buffers and solutions:

Table 3.1 Composition of media and broth used for adherence assays.

Media/Broth	Composition
5% Blood agar plate	15 g pancreatic digest of casein; 15 g papaic digest of soy meal; 5 g NaCl; 15 g agar; Bring to volume of 1 l and autoclave Adjust pH to 7.3 Cool to 45-50 °C; add 5% (v/v) sterile defibrinated blood; mix
MH broth	2 g dehydrated beef infusion; 17.5 g casein hydrolysate; 1.5 g starch; Bring to volume of 1 l and autoclave Adjust pH to 7.4

Table 3.2 Composition of buffers and solutions used for adherence assays.

Buffer/Solution	Composition
1× PBS	8 g NaCl; 0.2 g KCl; 1.44 g Na_2HPO_4; 0.24 g KH_2PO_4 in 800 ml dH_2O Adjust pH to 7.4 Add H_2O to 1 l and autoclave
1% HSA/PBS	1:20 of 20% HSA in PBS
2% HSA/PBS	1:10 20% HSA in PBS
0.1% crystal violet (500 ml)	3.75 g crystal violet; 250 ml ethanol (100%); 1.25 g NaCl; 24 ml formaldehyde (36.5%); 226 ml dH_2O
Elution buffer (500 ml)	25 ml 20% SDS; 475 ml PBS

3.4 Results

3.4.1 Adherence per ligand:

We were able to determine the adherence potential of all selected representative isolates to all different immobilised ligands tested, as well as an uncoated plate.

Figures 3.2 - 3.8 illustrate the adherence of individual isolates for the uncoated plate and the six specific immobilised ligands. For all seven figures, isolates have been grouped into their respective MLST CC and displayed according to HIV status (HIV negative; HIV status unknown; HIV positive (associated) [these two clones are statistically associated with HIV+ status] or HIV positive (isolated) [these two strains were collected from HIV+ persons]). Please refer to Appendix B (Figures B1 – B7) for a representation of the adherence of each individual isolate for the uncoated plate and the plates coated with immobilised ligands.

All isolates tested displayed variability in their adherence, irrespective of the ligand tested. Some isolates displayed a preference for adherence to immobilised fibronectin and fibrinogen, such as the isolates from CC8 and CC5. The CC30, CC15 and ST239 isolates displayed preference towards immobilised collagen IV and collagen VI. Some isolates were also identified to be poor binders to the immobilised ligands used, such as the ST88, ST97 and ST45 isolates. MRSA isolates displayed stronger adherence to plasma ($p = 0.025$), while no statistical associations were identified between adherence and patient HIV status or bacterial clonality. PVL- isolates displayed stronger adherence to fibronectin as compared to their PVL+ counterparts ($p = 0.034$).

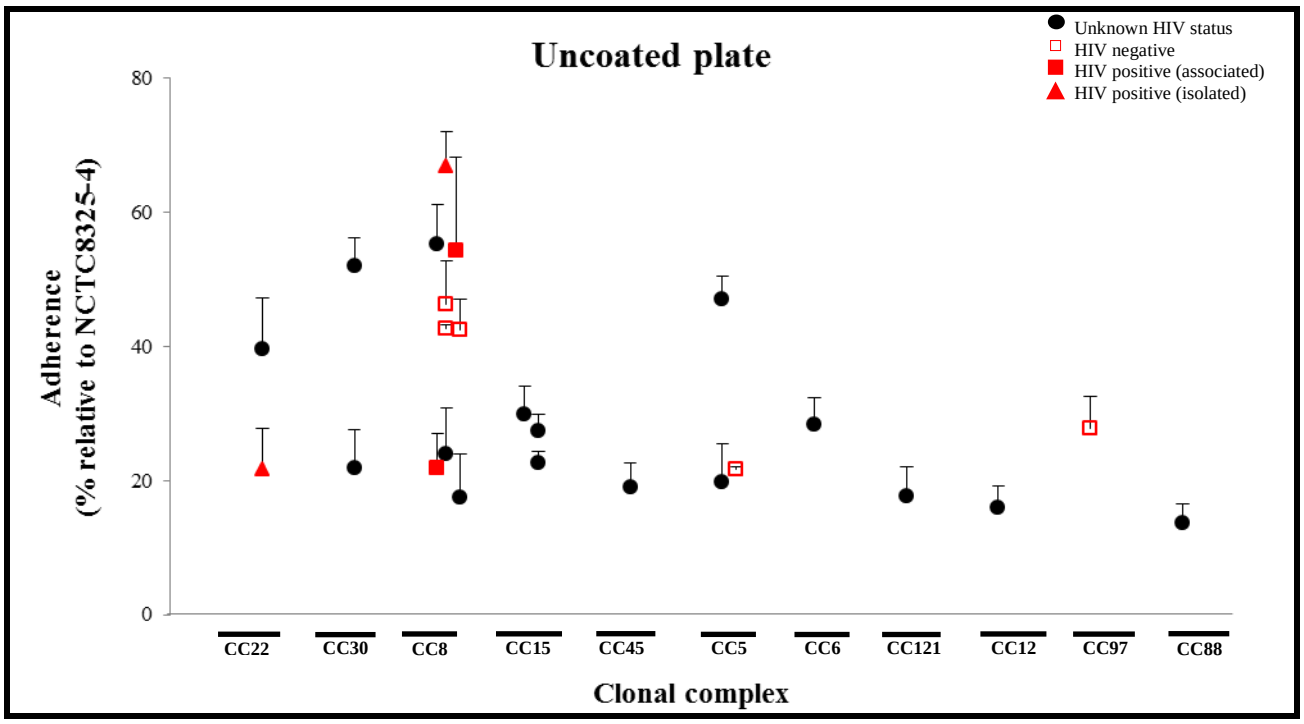


Figure 3.2 Relative adherence of isolates using an uncoated 96-well plate.

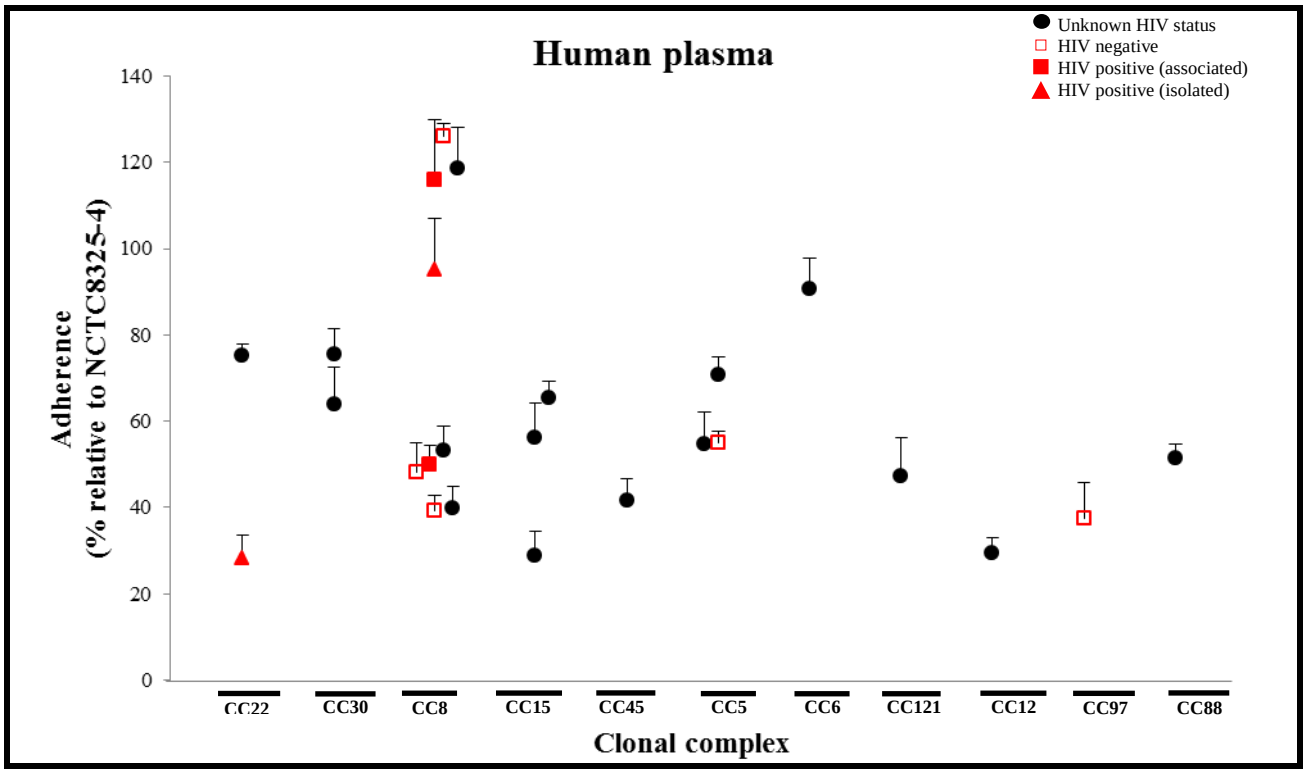


Figure 3.3 Relative adherence of isolates using a 96-well plate coated with human plasma proteins.

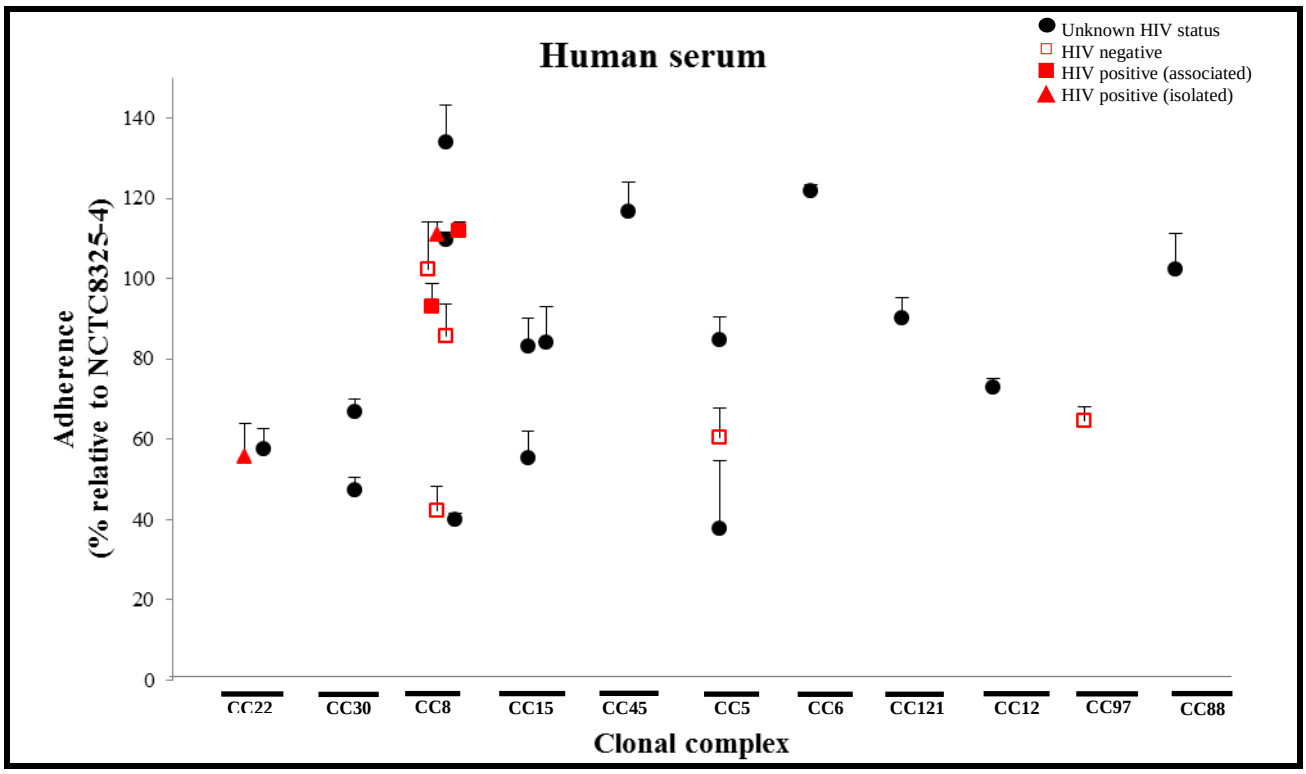


Figure 3.4 Relative adherence of isolates using a 96-well plate coated with human serum proteins.

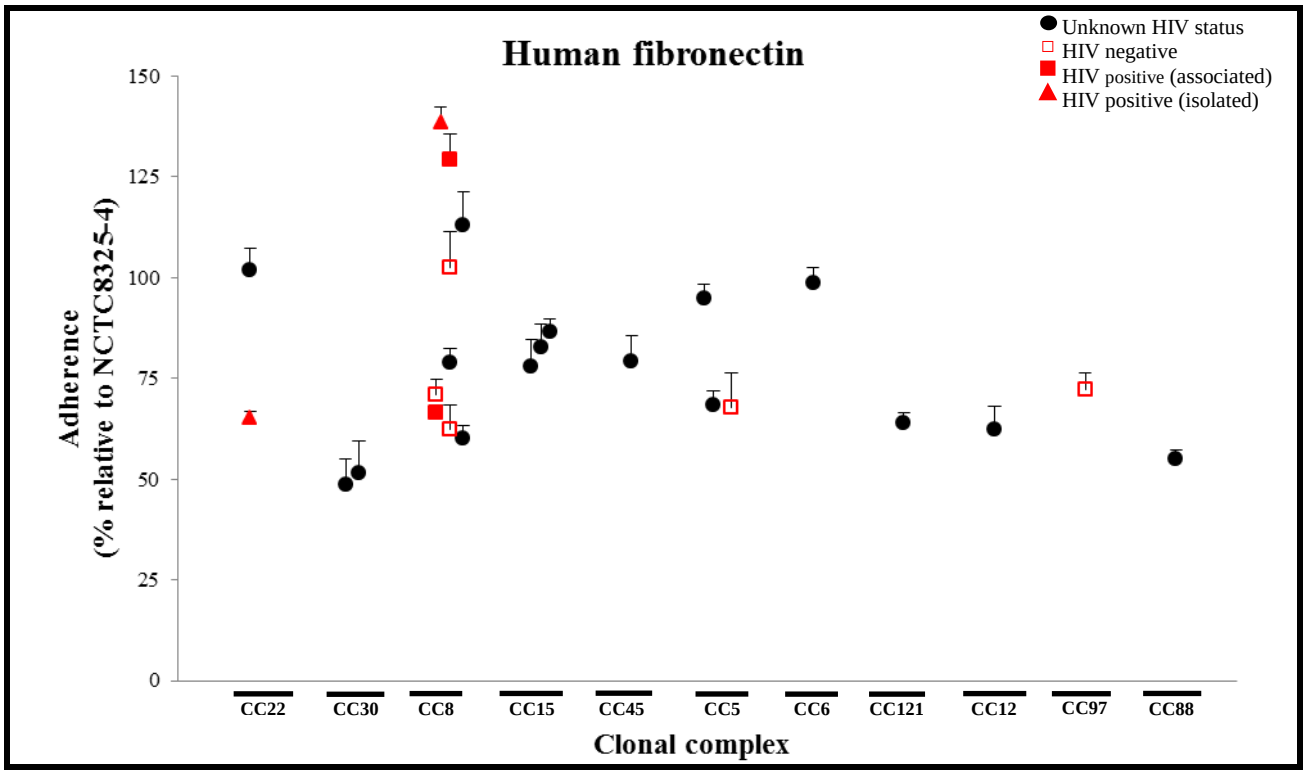


Figure 3.5 Relative adherence of isolates using a 96-well plate coated with human fibronectin.

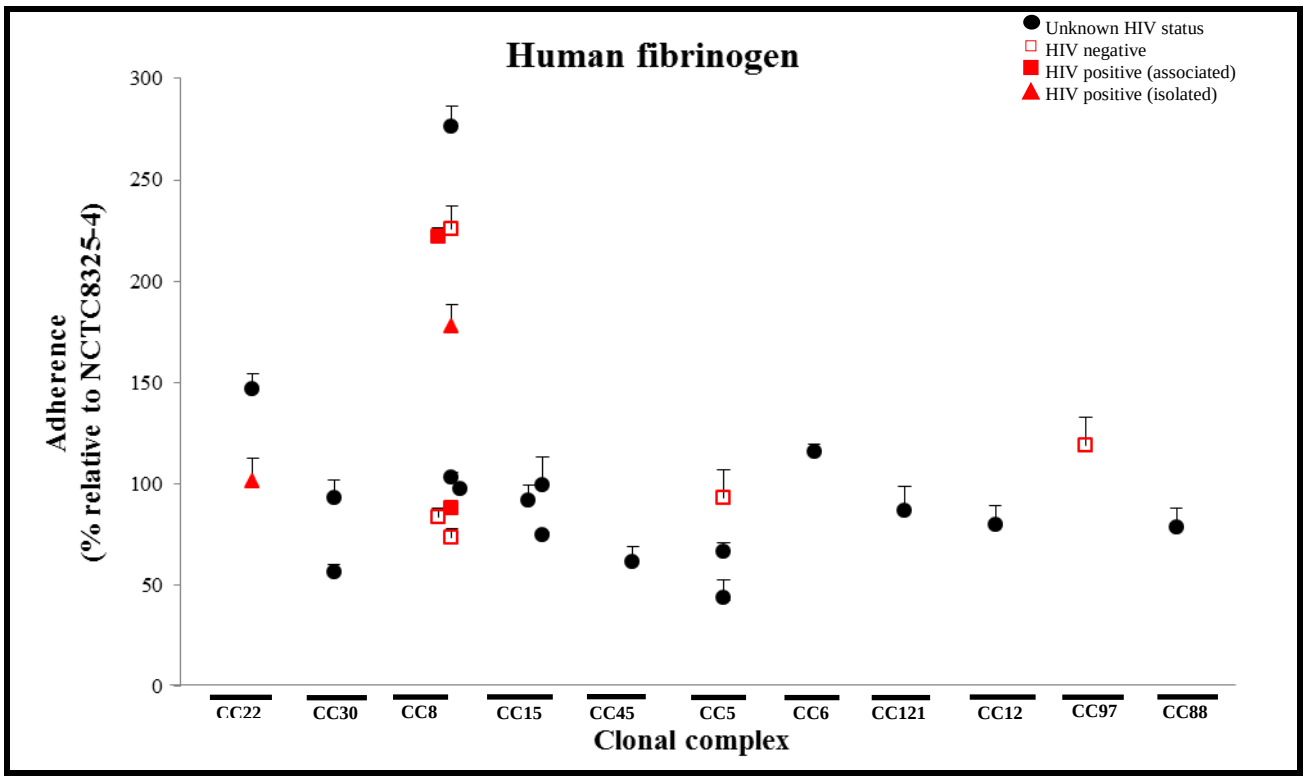


Figure 3.6 Relative adherence of isolates using a 96-well plate coated with human fibrinogen.

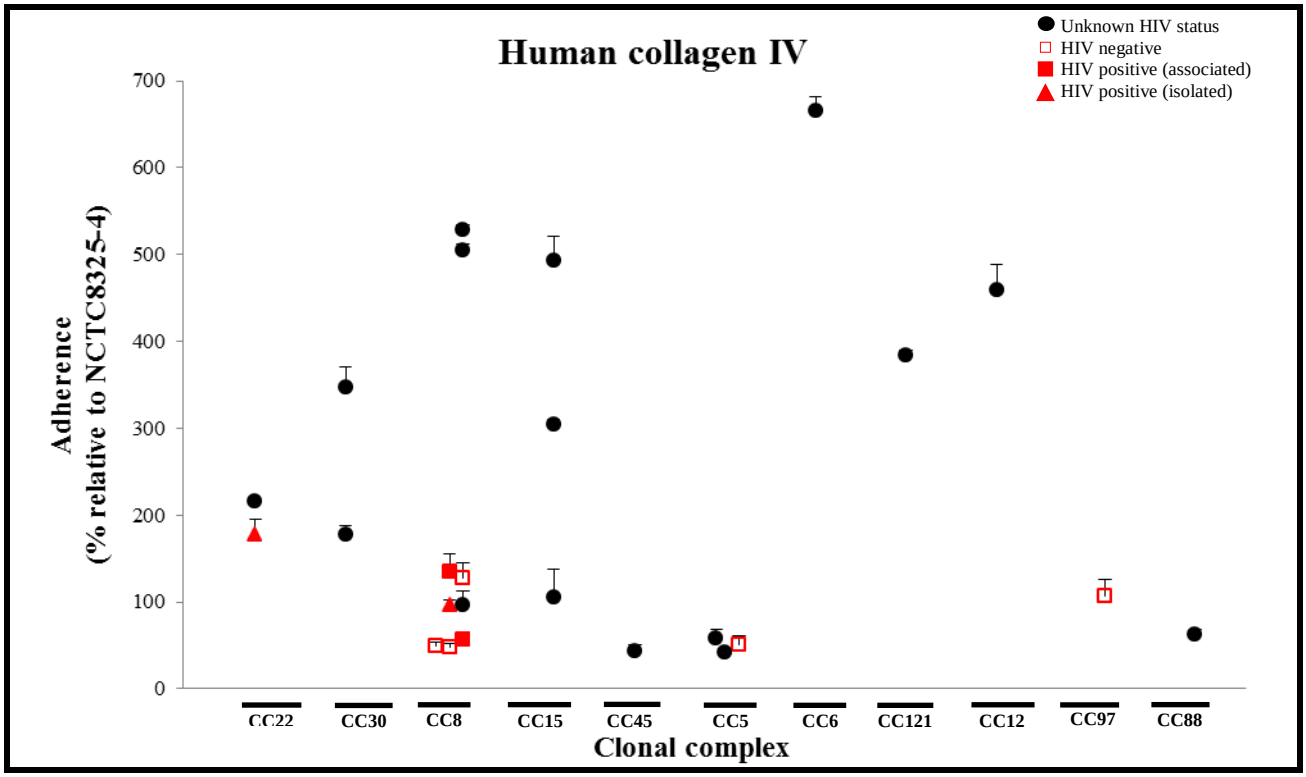


Figure 3.7 Relative adherence of isolates using a 96-well plate coated with human collagen IV.

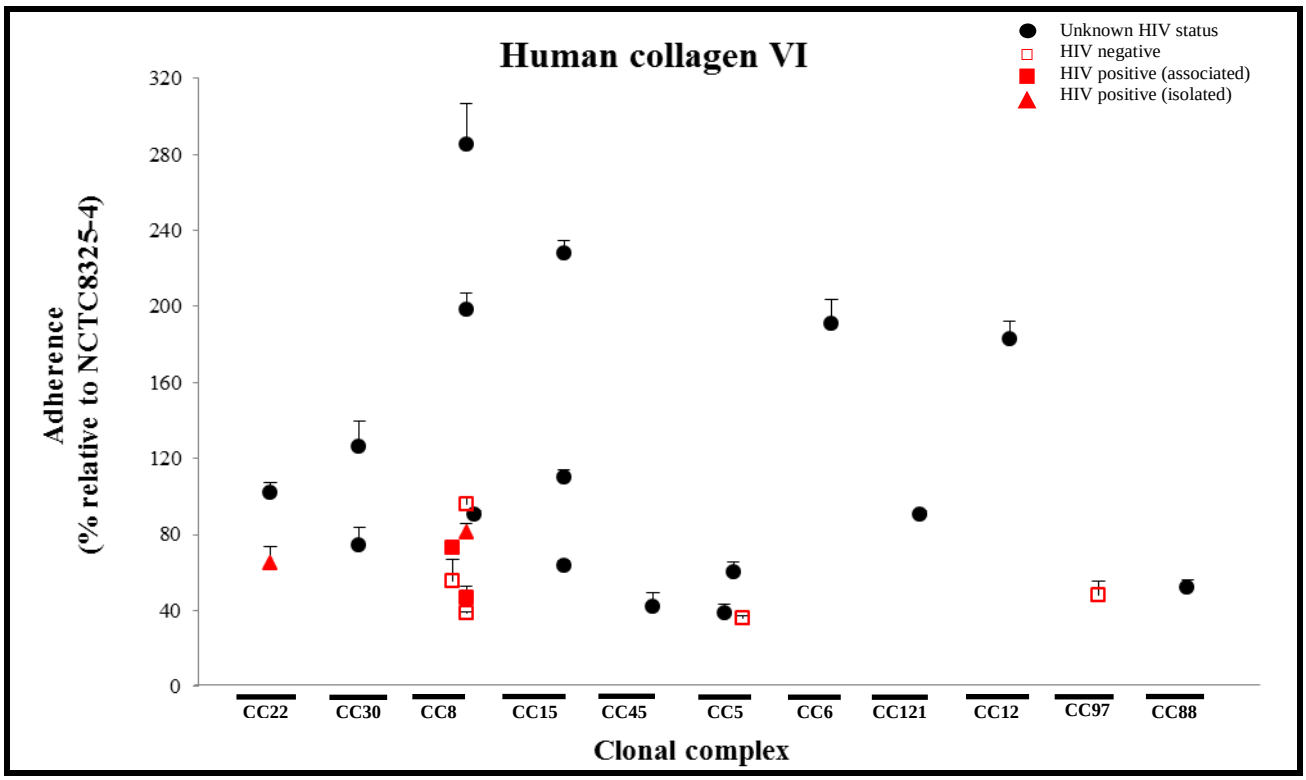


Figure 3.8 Relative adherence of isolates using a 96-well plate coated with human collagen VI.

3.4.2 Statistical associations:

We were able to identify that MRSA isolates adhered stronger in the absence of a ligand ($p = 0.016$) and to plasma proteins ($p = 0.025$) when compared to their MSSA counterparts, as displayed in Table 3.3. No statistical associations were identified between adherence and clonality, which is displayed in Table 3.4, or between adherence and patient HIV status (Table 3.5).

Table 3.3 Statistical associations between adherence and methicillin-resistance using the Mann-Whitney and 2-sample t-tests. Statistically significant values are displayed in bold ($p < 0.05$).

Ligand	Mann-Whitney test		2-sample t-test	
	Association with methicillinresistance	**p-value**	**Association with methicillin-resistance**	**p-value**
Uncoated	MSSA/MRSA	0.767	**MRSA**	**0.016**
Serum	MSSA/MRSA	0.613	MSSA/MRSA	0.365
Plasma	MSSA/MRSA	0.767	**MRSA**	**0.025**
Fibronectin	MSSA/MRSA	0.567	MSSA/MRSA	0.335
Fibrinogen	MSSA/MRSA	0.680	MSSA/MRSA	0.080
Collagen IV	MSSA/MRSA	0.440	MSSA/MRSA	0.505
Collagen VI	MSSA/MRSA	0.553	MSSA/MRSA	0.758

Table 3.4 Statistical associations between adherence and clonality using the Kruskal-Wallis test.

Ligand	Kruskal-Wallis test	
	Association with clonality	**p-value**
Uncoated	No association	0.194
Serum	No association	0.152
Plasma	No association	0.530
Fibronectin	No association	0.201
Fibrinogen	No association	0.160
Collagen IV	No association	0.248
Collagen VI	No association	0.292

Table 3.5 Statistical associations between adherence and patient HIV status using the Kruskal-Wallis test.

Ligand	Kruskal-Wallis test	
	Association with HIV status	**p-value**
Uncoated	No association	0.322
Serum	No association	0.506
Plasma	No association	0.849
Fibronectin	No association	0.581
Fibrinogen	No association	0.210
Collagen IV	No association	0.104
Collagen VI	No association	0.055

We were also able to identify that PVL-negative isolates adhered stronger in the absence of a ligand ($p = 0.010$) and to fibronectin ($p = 0.034$) when compared to the PVL-positive isolates, which is displayed in Table 3.6.

Table 3.6 Statistical associations between adherence and bacterial PVL status using the Mann-Whitney test. Statistically significant values are displayed in bold.

Ligand	Mann-Whitney test	
	Association with PVL status	**p-value**
Uncoated	**Negative**	**0.010**
Serum	No association	0.364
Plasma	No association	0.250
Fibronectin	**Negative**	**0.034**
Fibrinogen	No association	0.468
Collagen IV	No association	0.364
Collagen VI	No association	0.904

3.5 Discussion

Regarding associations made for the remainder of this chapter regarding adherence being a strain-specific or clonal trait, it is worth noting that these associations are made based on a small number of isolates included for the different MLST clonal complexes, while CC8 is over-represented. More representative isolates could not be included, as the techniques used for the *in vitro* characterisation are not high-throughput methods. The representative isolates were selected to represent each major and intermediate PFGE clones identified as described in Chapter 2 as well as minor clones based on clinical data (patient HIV status) and a specific bacterial characteristic (non-typeable SCC*mec*). Clonal complex 8 is over-represented among the selected representative isolates, mainly due to the inclusion of 2 isolates statistically associated with HIV-positive patients and 1 isolate from the HIV-positive patient from the dominant MRSA clone, ST612. The researchers were unable to make any deduction for CC45, CC6, CC121, CC12, CC97 and CC88 as only one isolate for each CC was included for further analyses.

The representative isolates selected displayed a diverse range of adherence to any specific immobilised ligand, for all ligands tested, as well as for adherence in the absence of a specific ligand. No significant difference was observed for adherence to any of the ligands tested, between isolates from HIV+ and/or HIV- persons. It is worth noting that genotypically, all representative isolates were positive for all proteins involved in adherence to the immobilised ligands tested, yet diversity was displayed during the *in vitro* adherence to the individual ligands.

3.5.1 Discussion per ligand:

Regarding adherence in the absence of a specific ligand (uncoated plate), our selection of representative clinical isolates displayed lower adherence compared to the positive control, with a median adherence of 31.9% (range 13.7 – 55.3%) of the reference strain. Isolates of CC22, CC30, CC8 and CC5 displayed a diverse range of adherence. In this instance, adherence for these CCs seems to be a strain specific characteristic, as opposed for CC15, where it seems to be a clonal trait. Please refer to Figures B1 and 3.2. A statistical association was only identified using the 2-sample t-test, which indicated that MRSA isolates adherence was stronger in the absence of a ligand, when compared to MSSA isolates ($p=0.016$) and is displayed in Table 3.3.

Isolates of CC8 again displayed the greatest range of adherence to human plasma proteins, adhering up to 126% relative to the positive control. Fibronectin and fibrinogen are components of the human plasma that are likely to act as ligands for bacterial adherence. The median adherence was 62.3% (range 28.4 - 126.1%), lower than the control, for this collection of isolates. Isolates of CC22, CC8 and CC15 adhere to human plasma proteins in a strain-specific manner. Adherence for CC30 and CC5 isolates seems to be a clonal trait. Please refer to Figures 3.3 and B2. Plasma was the only other ligand where a statistical association could be identified, also using the 2-sample t-test. Again, MRSA isolates adhered stronger to human plasma proteins, when compared to MSSA isolates ($p=0.025$). Please refer to Table 3.3., Section 3.4.2.

A similar picture is seen for human serum (fibronectin as common component), with CC8 isolates adhering up to 134% relative to the control. The median adherence was 81.2% (range 37.6 – 121.8%), lower than the control. Adherence to human serum proteins was a strain-specific characteristic for isolates of CC30, CC8, CC15 and CC5, while it was clone-specific characteristic for CC22 isolates. Please refer to Figures B3 and 3.4.

The median adherence to human fibronectin for this collection of isolates was 80% (range 48.5 – 138.5%), lower than the control. Isolates from CC8 isolates again displayed the greatest degree of adherence, adhering up to 138% relative to the control. Adherence to human fibronectin was a clonal trait for isolates of CC 30 and15 and a strain-specific trait for isolates of CC22, CC8 and CC5. Please refer to Figures B4 and 3.5. PVL-negative isolates adhered stronger to fibronectin than the PVL-positive isolates.

The majority of isolates displayed a significant ability to adhere to human fibrinogen, with a median adherence of 110% (range 43.8 – 276.2%) for this collection of isolates, higher than the control. Only isolates of CC15 displayed a clone-specific trait for adherence to human fibrinogen. Regarding isolates of CC22, CC30, CC8 and CC5, adherence was a strain-specific characteristic. Adherence for CC8 isolates ranged from 73.2% - 276.2%, where 5/9 isolates of this CC displayed adherence of >100%. Please refer to Figures B5 and 3.6.

The adherence profiles obtained for both collagens tested appeared similar to each other and yet very different from the previously discussed ligands. Strong adherence to at least collagen IV as an immobilised ligand was seen and strong adherence towards collagen VI was also noticed. The isolates displayed a very broad range of adherence for both collagens tested.

The median adherence was 213.3% for this collection of isolates for human collagen IV (range 41.3 – 528.7%). Although CC22 isolates adhered very strongly to collagen IV (mean 197%) and isolates of CC5 displayed a moderate adherence to collagen IV (mean 50%), adherence to collagen IV was a clonal trait for both CCs. Pertaining to eisolates of CC30, CC8 and CC15, adherence to collagen IV was a strain-specific characteristic. Adherence for isolates of CC30 ranged from 177% - 347%, while for CC15 at ranged from 105% - 493%. The isolates of CC8 could be re-grouped into smaller clusters based on adherence: cluster 1 contained MRSA isolates (adherence 505% - 528%), and cluster 2 contained both MRSA and MSSA isolates (adherence 48% - 135%). The strongest adherence to collagen IV was displayed by the ST6 isolate (665%), while the ST121 (383%) and the ST12 (458%) isolates also displayed very strong affinities to immobilised collagen IV. Please refer to Figures B6 and 3.7.

The median adherence for this collection of isolates for human collagen VI was 99% (range 36.1 – 285.3%), while a CC8 MRSA isolate displayed the strongest adherence to collagen VI (285%). Adherence to collagen VI was a clonal characteristic for CC5 isolates, while those of CC22, CC30, CC8 and CC15 displayed a strain-specific adherence preference towards this immobilised ligand. Two C8 MRSA isolates and one CC15 isolate displayed very strong adherence (200%) to collagen IV. The CC12 (183%) and the CC6 (191%) isolates also displayed strong adherence to collagen VI. Please refer to Figures B7 and 3.8.

3.5.2 Discussion per clonal complex:

Regarding the CC22 isolates, we see a strain-specific adherence pattern to all immobilised ligands, except for human serum, which is rich in fibronectin, and collagen IV. A different pattern is observed for purified human fibronectin, where THW146 (ST22-MSSA) display stronger adherence than THW393 (ST22-MSSA). Both isolates display a preference for human collagen IV (>175%) and fibrinogen (>100%), while THW146 only displays preference for collagen VI (>100%). Please refer to Figure 3.9.

Regarding the CC30 isolates, we see a strain specific adherence pattern for collagens IV and VI, fibrinogen and the uncoated plate. Adherence to fibronectin and human plasma (rich in fibronectin) seems to be a clonal trait. THW382 (ST36-MRSA-II) adheres strongly to collagen IV (>150%) and fibrinogen (90%). THW38 (ST1865-MSSA) displayed extremely strong adherence to collagen IV (350%) and strong adherence towards collagen VI (130%). Please refer to Figure 3.10.

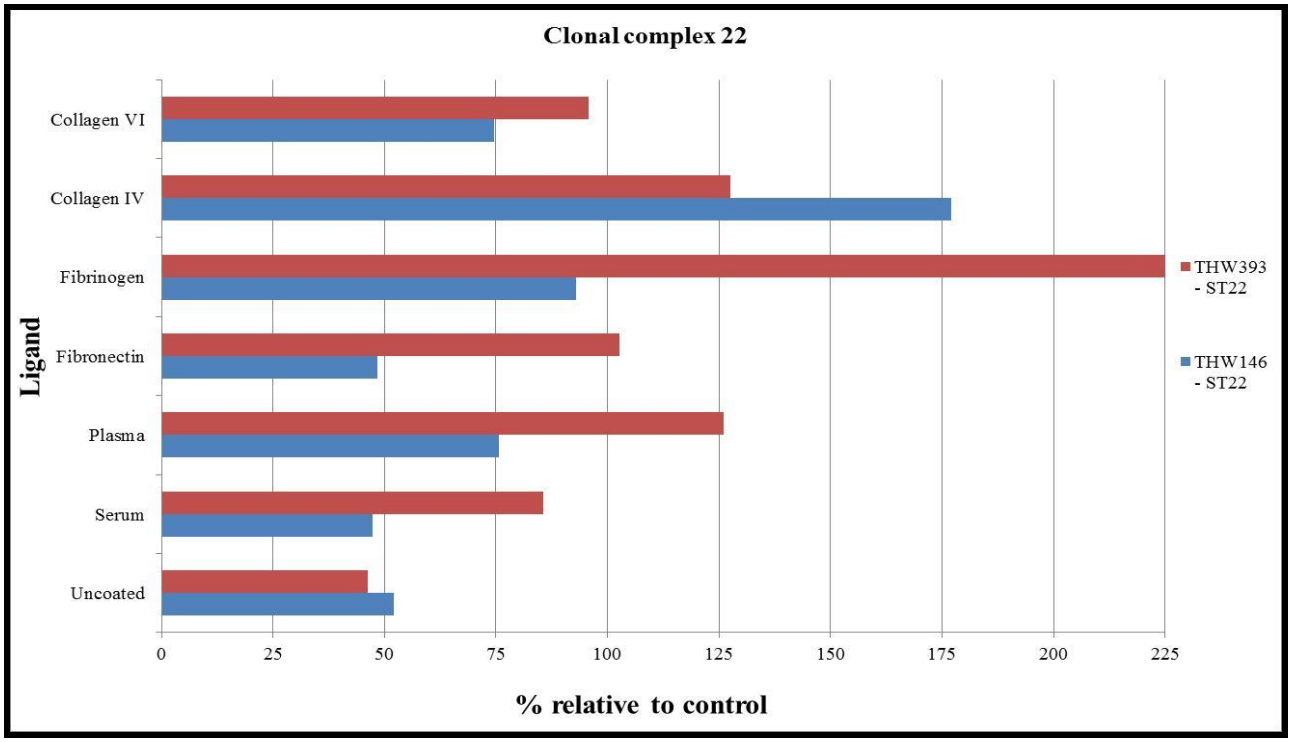


Figure 3.9 Adherence of CC22 isolates in the absence/presence of various immobilised human ligands.

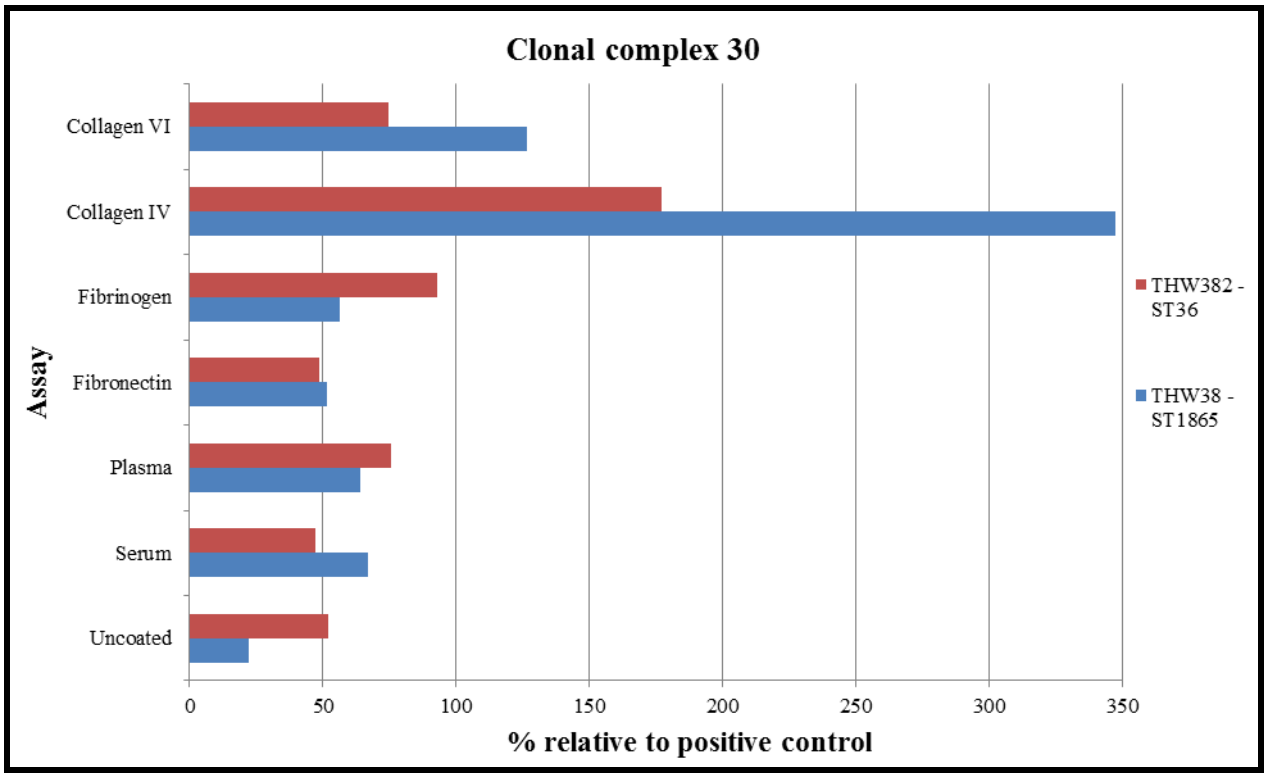


Figure 3.10 Adherence of CC30 isolates in the absence/presence of various immobilised human ligands.

Regarding the CC8 isolates, we see a general diverse pattern of adherence, which seems to be a strain-specific characteristic for all ligands tested. Interestingly, both ST239 isolates, THW99 (ST239-MRSA-NT) and THW81 (ST239-MRSA-III), display very strong adherence to collagen VI (200-300%) and extremely strong adherence to collagen VI (>500%). These isolates also display strong adherence to fibrinogen (100%) and only moderate adherence to fibronectin and plasma (50%). THW81 displays relatively strong adherence to serum (> 100%) while THW99 does not. Please refer to Figure 3.11.

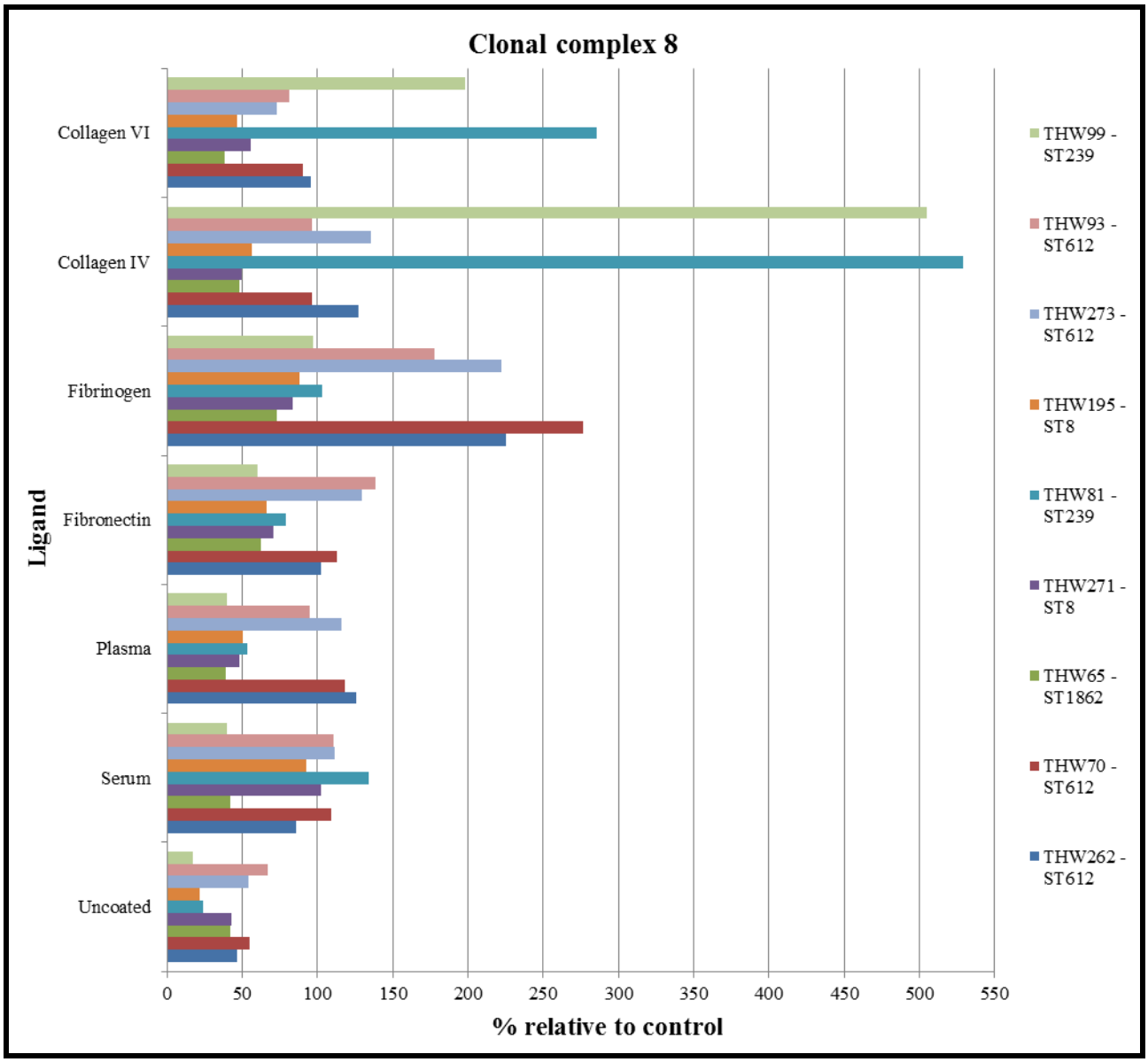


Figure 3.11 Adherence of CC8 isolates in the absence/presence of various immobilised human ligands.

All four ST612 isolates (THW93, 273, 70 and 262 – ST612-MRSA-IV) display strong to very strong adherence to fibrinogen (175-275%) and also strong adherence to fibronectin (100-140%) and plasma (100-125%) (Figure 3.11). ST612 was the dominant MRSA clone identified and has previously been reported in Australia[117] and South Africa[120] only. Fibronectin may play a role in SSTI and 2/4 isolates were classified as SSTI by source. Fibrinogen and fibronectin co-operate for heart valve/endothelial infection, thus making the ability to adhere strongly to these two ligands essential for establishing infective endocarditis[84].

Regarding the isolates of CC15, adherence to fibrinogen, plasma and serum seems to be a strain specific trait and is moderate to high. Adhere to fibronectin is relatively high and seems to be a clonal trait. Adherence to the collagens also seems to be a strain specific trait. Adherence of THW224 (ST188-MSSA) and THW412 (ST1-MSSA) to collagen VI is strong (100-200%) and to collagen IV is very strong (300-500%). Please refer to Figure 3.12.

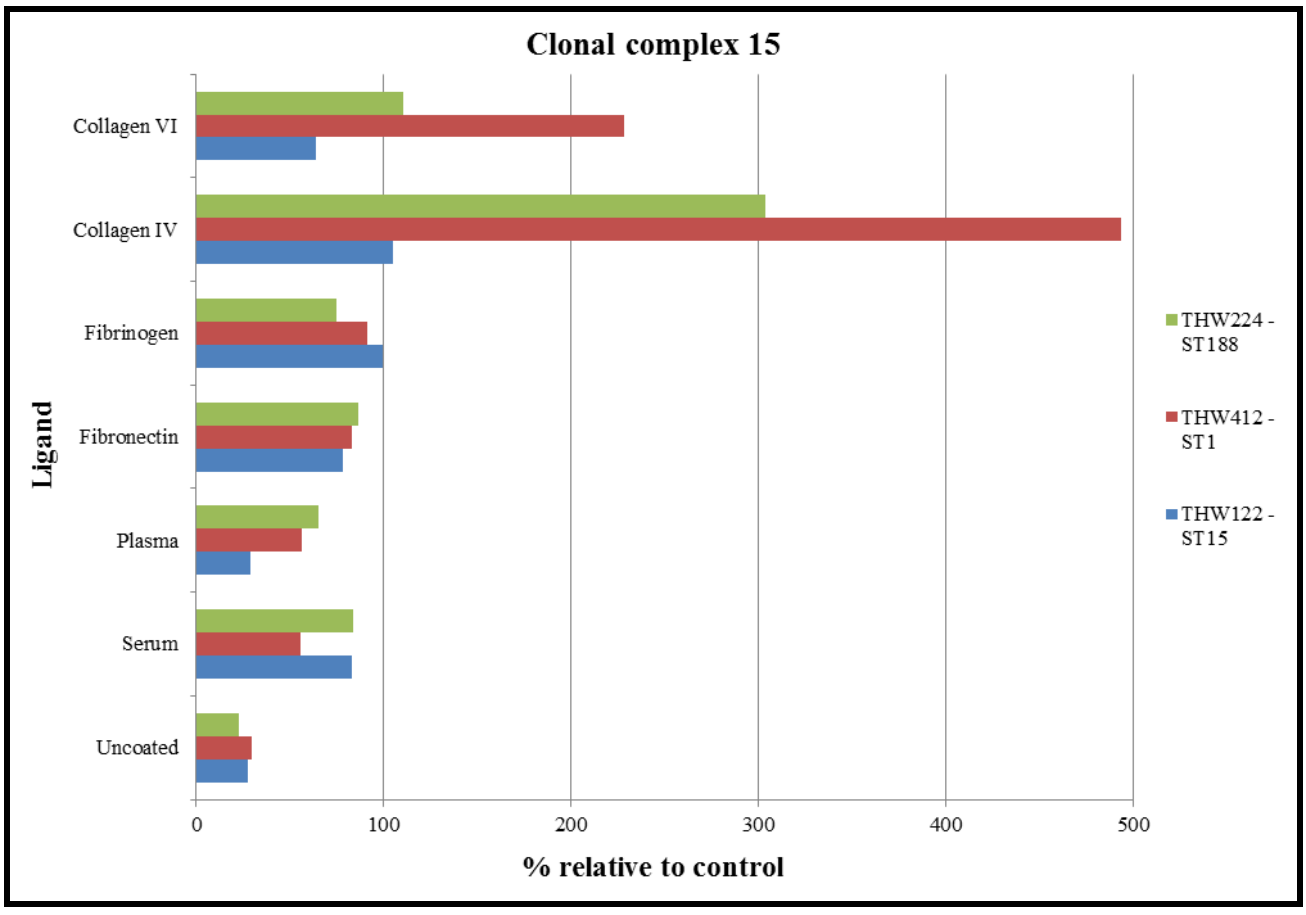


Figure 3.12 Adherence of CC15 isolates in the absence/presence of various immobilised human ligands.

Regarding the isolates of CC5, adherence in general is low to moderate and appears to be a strain specific trait for all ligands tested. THW17 (ST1863-MSSA) display relatively strong adherence to fibronectin and serum (80-90%) and also the strongest adherence to both the collagens (60-70%), although adherence is only moderate. THW235 (ST1864-MSSA) adhere relatively strong to fibrinogen (80%), while THW264 adhere the strongest adherence to plasma proteins and to no ligand (uncoated plate). Please refer to Figure 3.13.

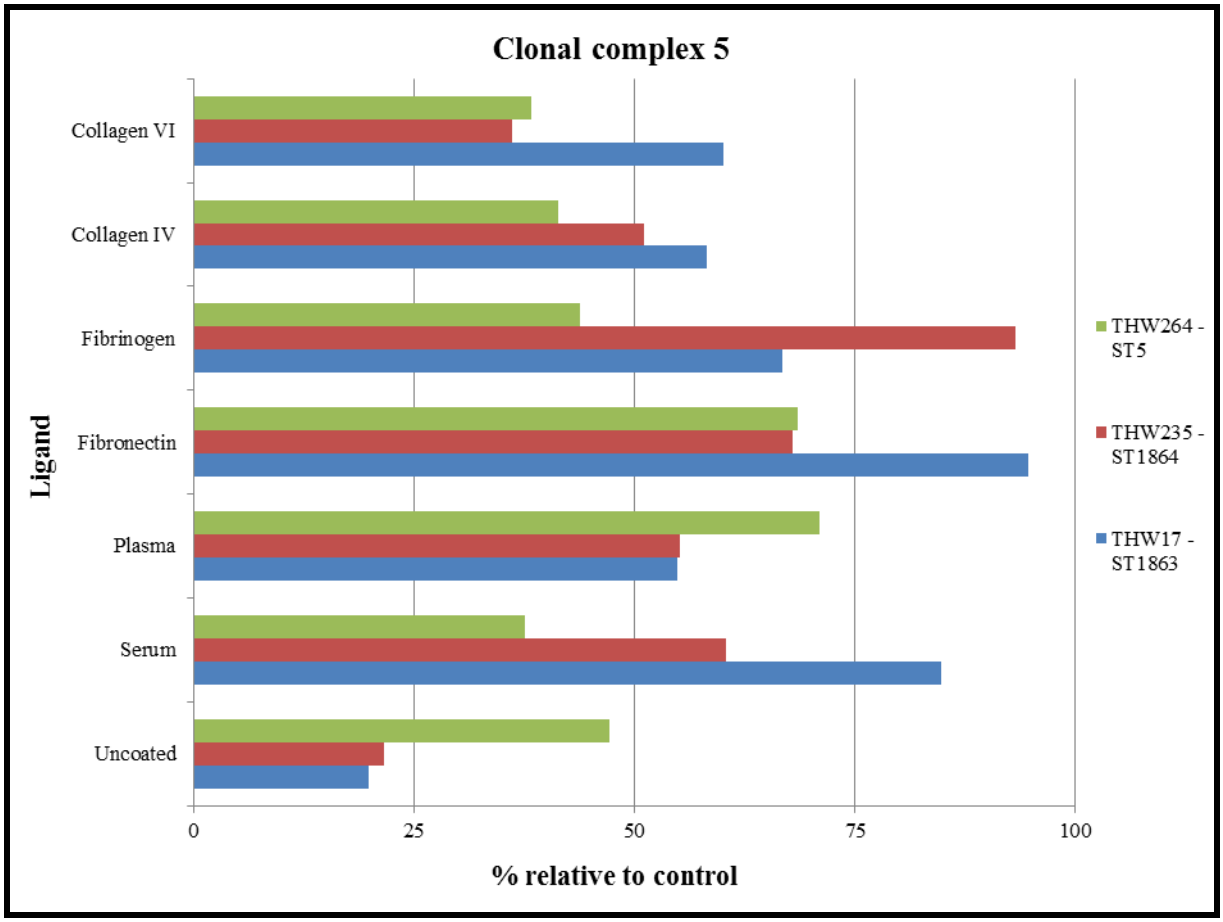


Figure 3.13 Adherence of CC5 isolates in the absence/presence of various immobilised human ligands.

Regarding the singletons, we cannot make any deductions regarding adherence being a strain or clonal trait as only one isolate was analysed for every ST. THW368 (ST12-MSSA), THW366 (ST121-MSSA) and THW64 (ST6-MSSA) displayed strong preference for adherence towards the collagens, adhering strongly to collagen VI (100-200%) and very strongly to collagen IV (400-700%). The THW64 isolate also displayed strong adherence towards fibronectin, fibrinogen, plasma and serum (100% all). THW255 (ST88-MRSA-IV) and THW356 (ST45-MSSA) adhered

strongly to serum only (100%), while the THW241 (ST97-MSSA) displayed strong adherence towards collagen IV and fibrinogen (100%). Please refer to Figure 3.14.

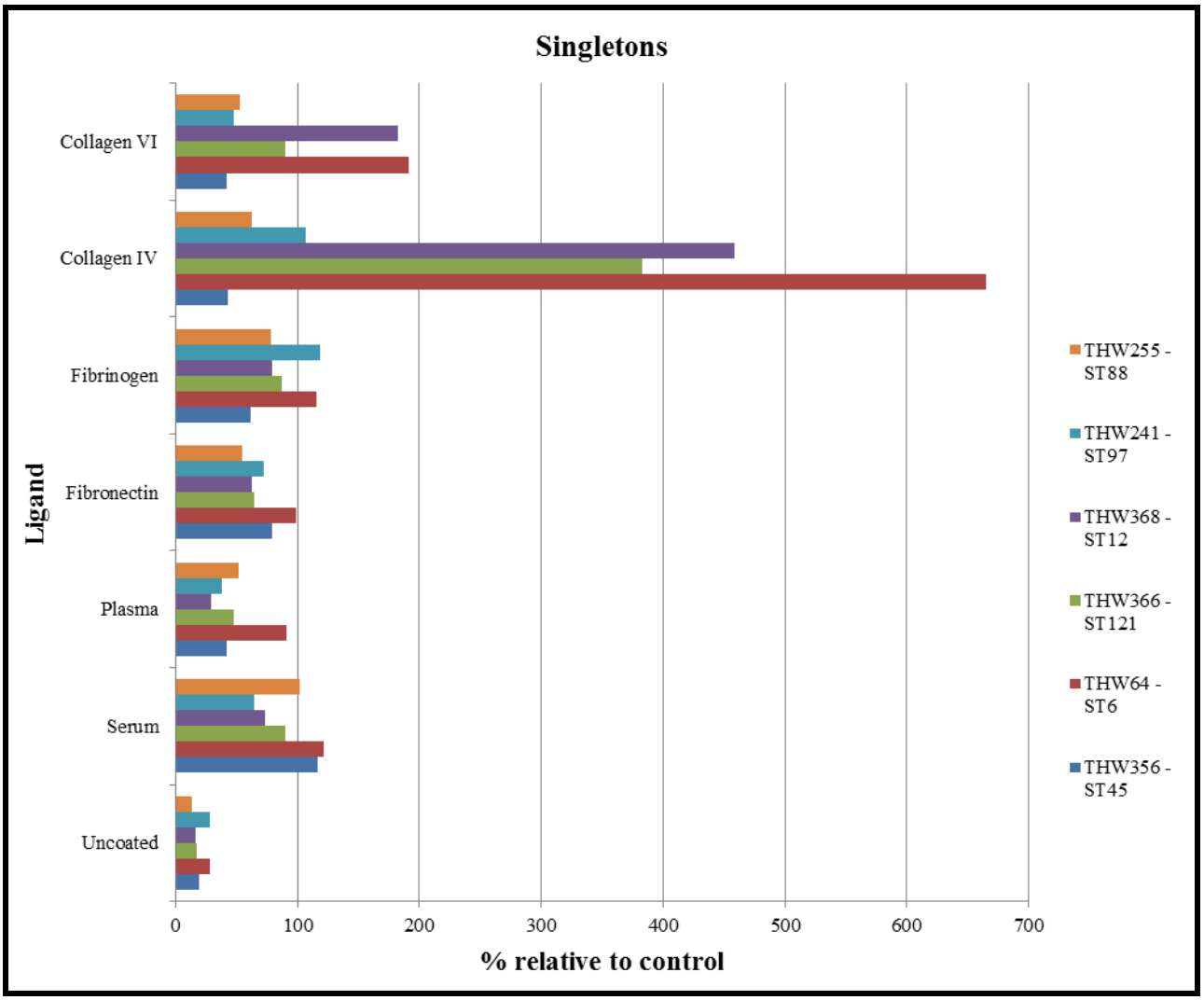


Figure 3.14 Adherence of various singleton STs in the absence/presence of various immobilised human ligands.

No statistical association was identified between any of the ligands and clonality. The presence of both *fnb* genes found in this collection is similar to what has been previously reported for isolates collected from orthopaedic implants[260].

As previously shown by Peacock *et al*[163], this collection of *S. aureus* isolates displayed diversity in adherence to human fibronectin, but not in the genotypic presence of *fnb* genes. In contrast to the Peacock study, all selected representative isolates tested PCR-positive for both *fnb*A and *fnb*B.

Unfortunately, no data is available on the expression of these two genes for this study. Adherence to fibronectin is thus achieved by more than one adhesin working simultaneously, and is significantly involved in bacterial adherence to the endothelium *in vivo*[261].

Elgalai *et al.* also described a collection of wound isolates that displayed a diverse range of adherence and similarly to our set of isolates, displayed stronger adherence to fibrinogen than to fibronectin.[262]. However, this set displayed stronger adherence to collagen IV. Seidl *et al.* described no significant adherence potentials to immobilised fibronectin between isolates derived from persistent and resolving MRSA infections[263].

Unlike previously reported by Arciola *et al.*[260] for orthopaedic implant infections, adherence to both human collagen IV and VI varied greatly among the isolates, irrespective of the presence or absence of the *cna* gene, while Campoccia *et al.* referred to *cna*, together with bone sialoprotein-binding protein (encoded by the gene *bbp*) as crucial virulence factors in the pathogenesis of orthopaedic implant infections[264]. This contradicts the work of Thomas *et al*[265]. Hartford *et al.* described the adherence of *cna* mutants to immobilised collagen and found that mutants of the A domain of *cna* bound lower to the immobilised proteins than wild-type strains[266]. De Bentzman *et al.* describe a collection of isolates clinically confirmed to be community-acquired necrotising pneumonia (NP) or non-necrotising pneumonia, where the PVL+ NP (cna^+) isolates displayed stronger adherence towards collagen IV[267] when compared to the PVL- NP (cna^-) isolates.

Indirect evidence by serology suggests that both *clf*A and *clf*B are produced *in vivo* and are important factors for valve infection for the promotion of infective endocarditis[268] and were expressed by numerous isolates associated with this disease. This collection of isolates displayed strong adherence for fibronectin, followed by fibrinogen even though no blood culture isolates were included in this collection.

Hansen *et al.* described Eap as a protein which binds "promiscuously to monomeric matrix molecules" while selectively recognising extracellular matrix aggregates[250]. There data obtained using recombinant Eap (rEap) indicated that the rEap preferred adherence to fibronectin, followed by collagen IV and collagen VI, while the organism adhered at low numbers to collagen II and virtually nothing to collagen I. The researchers also found that microfibrils containing collagen IV, collagen VI rich baseman membrane-associated networks and fibronectin-containing aggregates adhered to Eap as affectively as the monomeric immobilised ligands[250].

3.6 Conclusion

Clinical *S. aureus* isolates collected at Tygerberg hospital display diversity in their abilities to adhere to various immobilised human ligands.

Some isolates of certain clonal complexes displayed strong adherence to the two types of collagen tested, even stronger than for fibronectin or fibrinogen (on a relative level). MRSA isolates also displayed stronger adherence to human plasma proteins than MSSA isolates, while PVL- isolates adhered stronger to fibronectin.

3.7 Appendix B

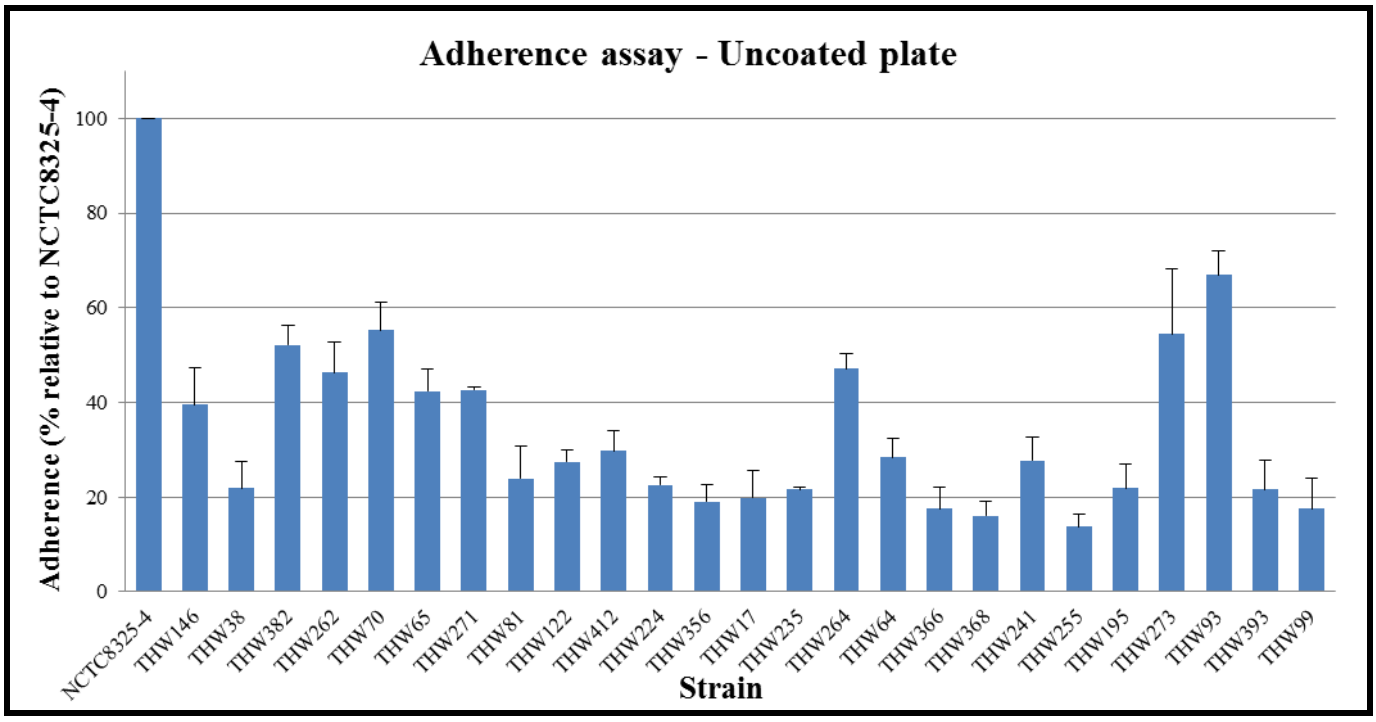


Figure B1 Relative adherence of isolates using an uncoated 96-well plate.

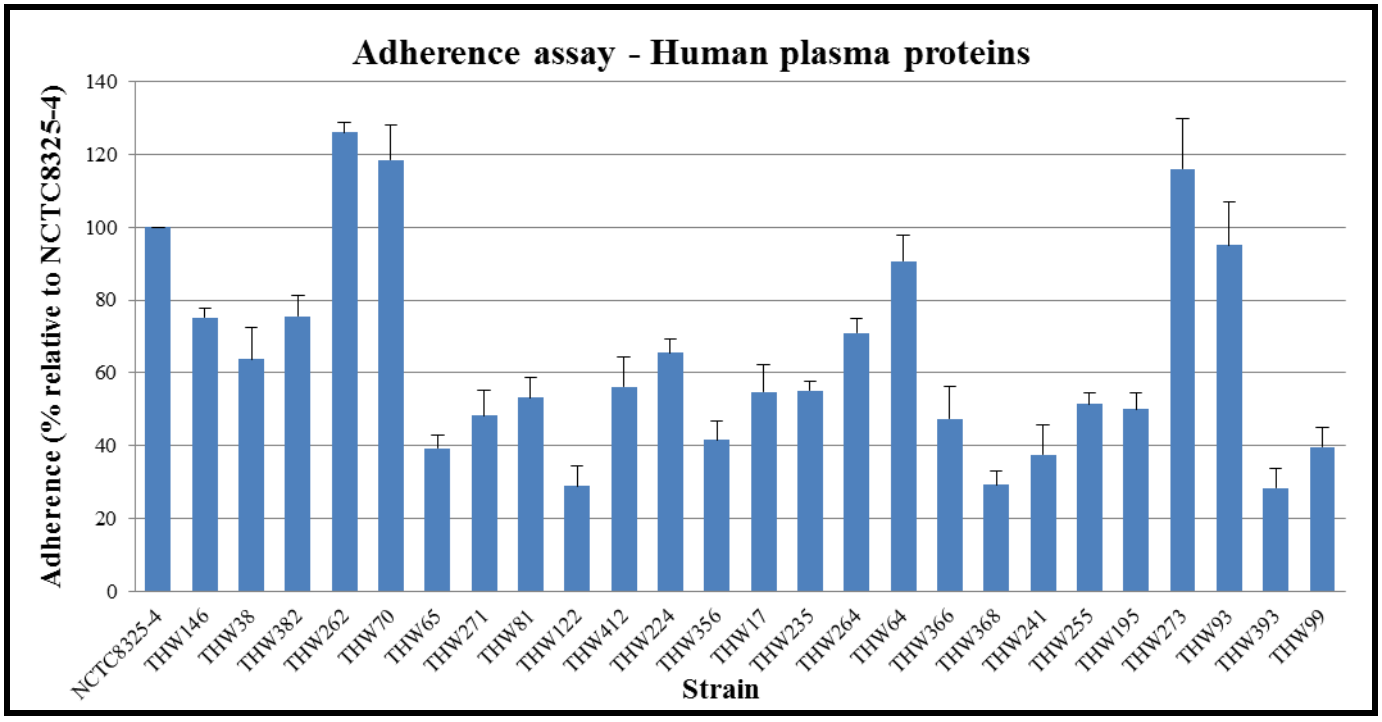


Figure B2 Relative adherence of isolates using a 96-well plate coated with human plasma proteins.

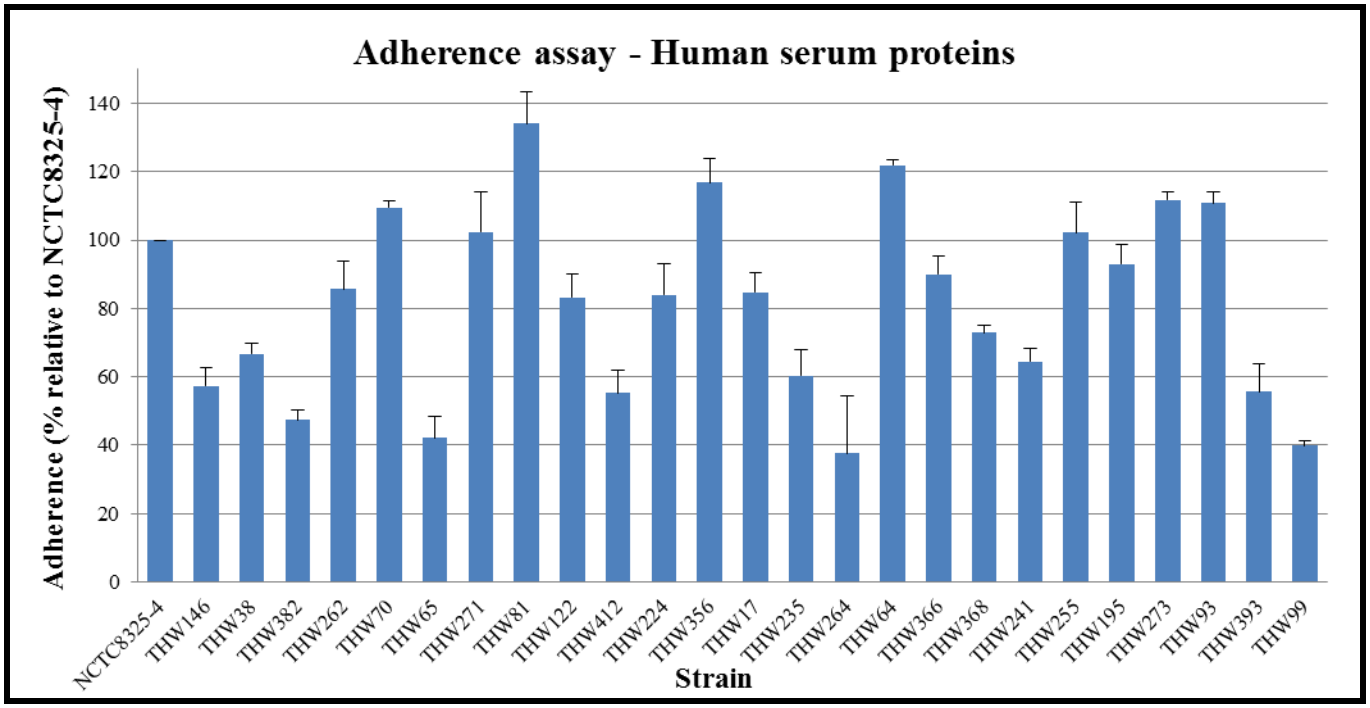


Figure B3 Relative adherence of isolates using a 96-well plate coated with human serum proteins.

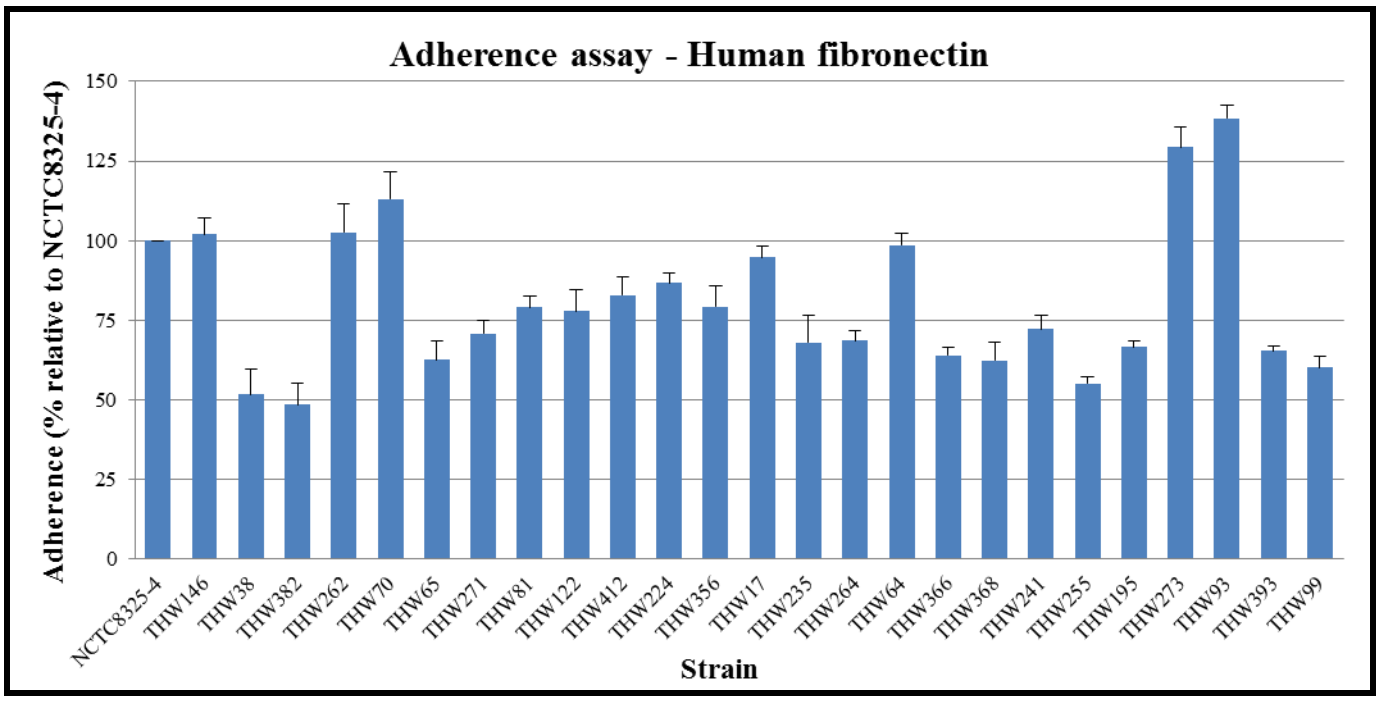


Figure B4 Relative adherence of isolates using a 96-well plate coated with human fibronectin.

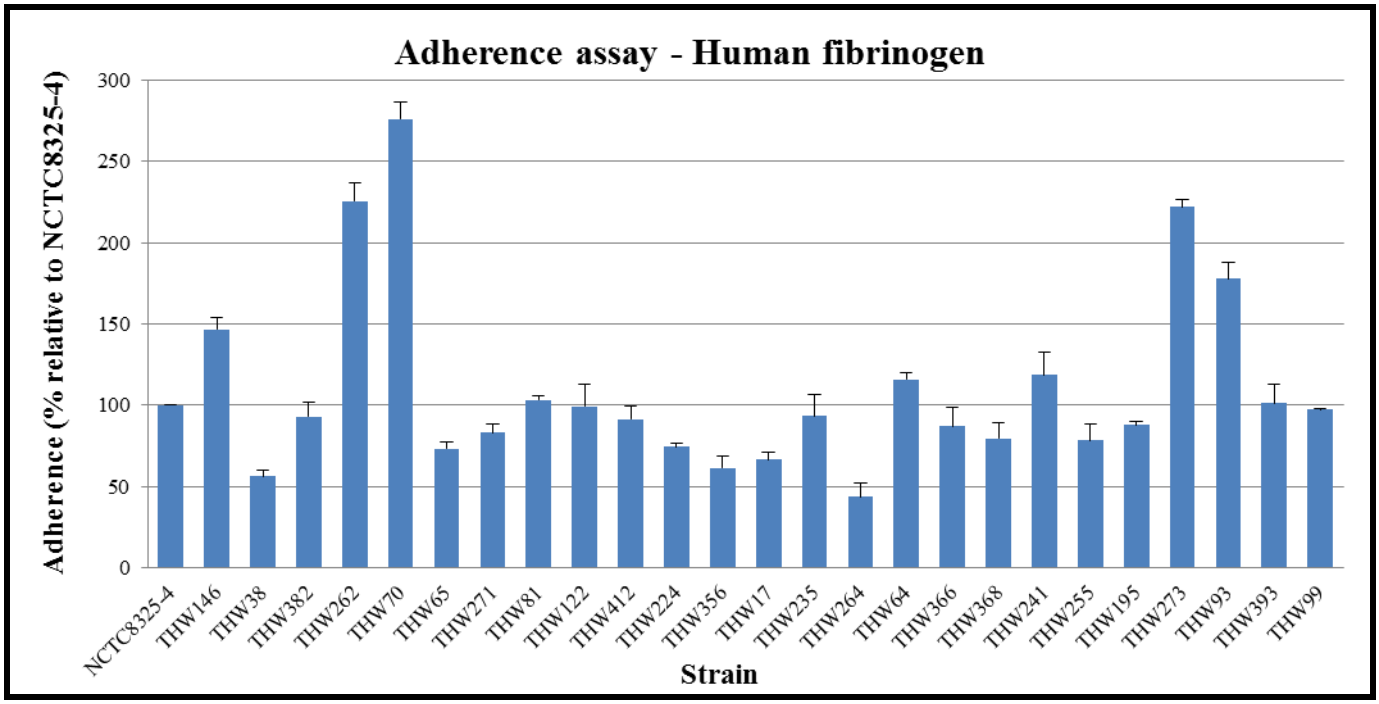


Figure B5 Relative adherence of isolates using a 96-well plate coated with human fibrinogen.

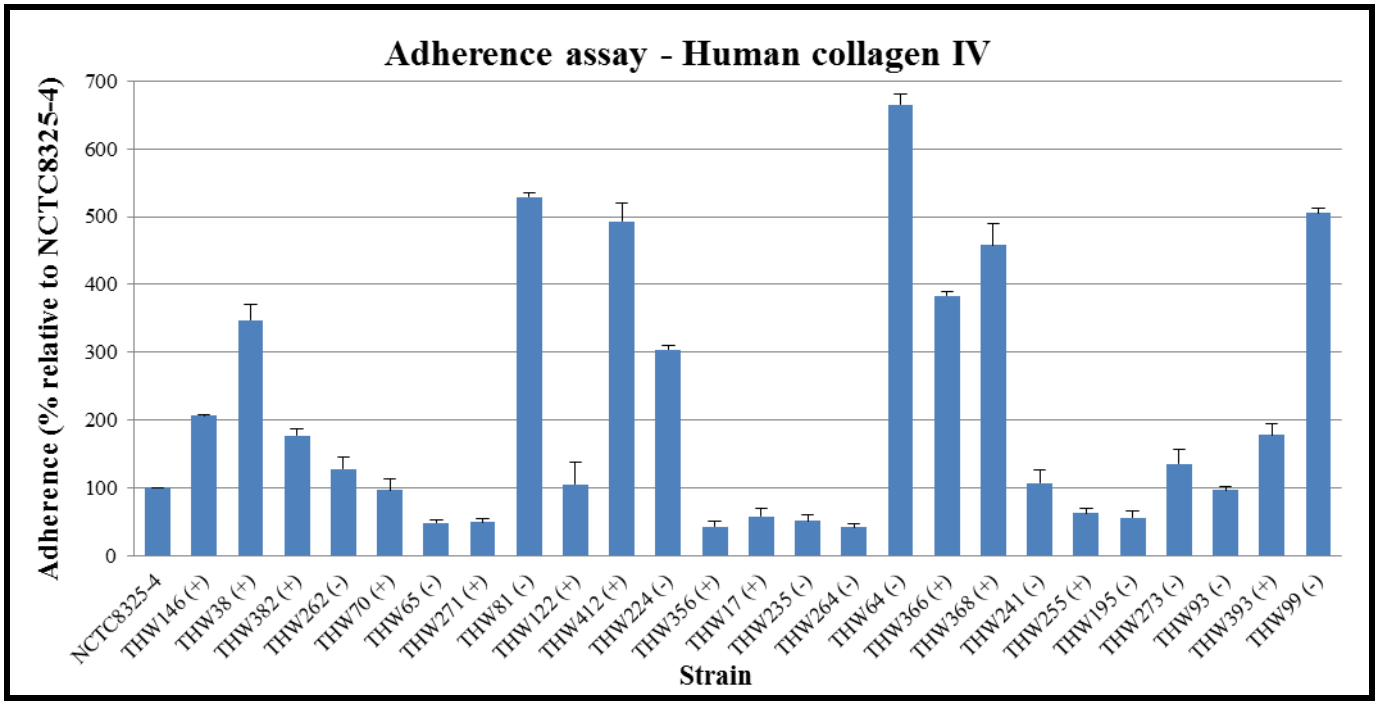


Figure B6 Relative adherence of isolates using a 96-well plate coated with human collagen type IV. +/- indicate presence/absence of *cna* gene.

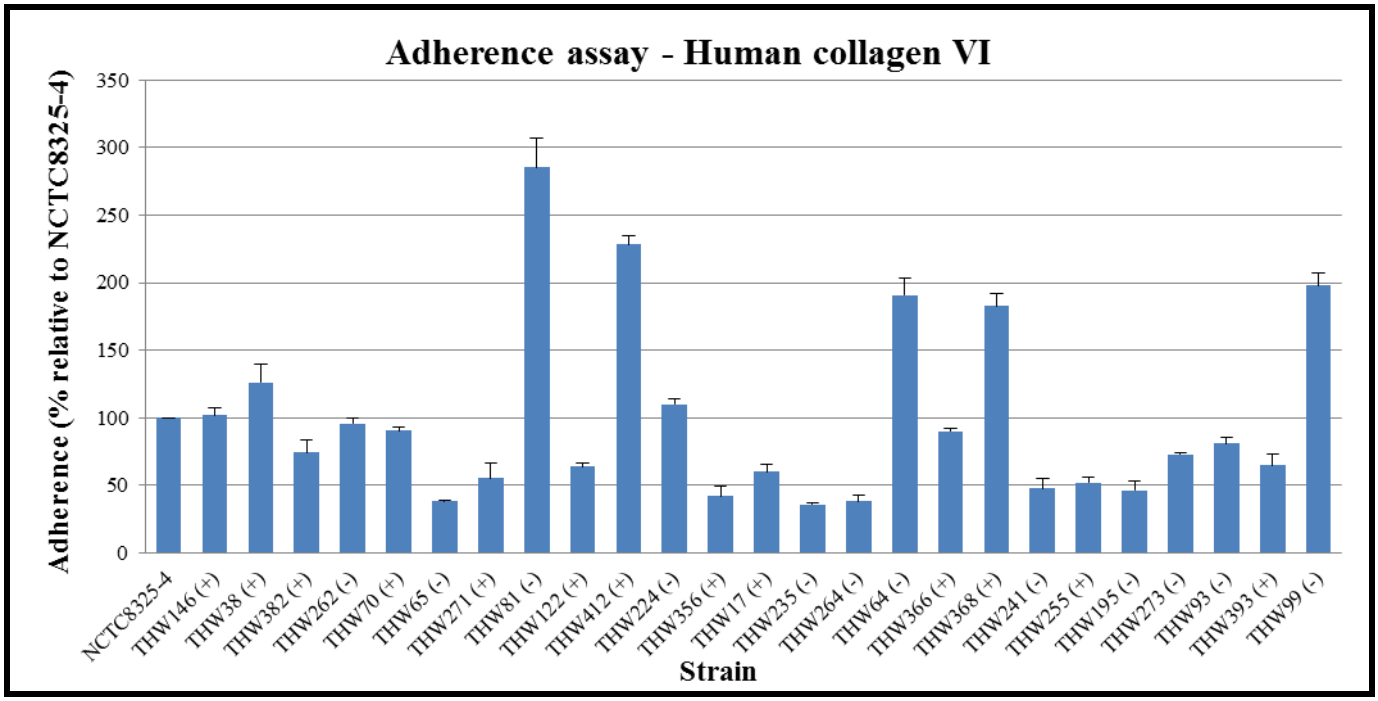


Figure B7 Relative adherence of isolates using a 96-well plate coated with human collagen type VI. +/- indicate presence/absence of *cna* gene.

Table B1 Strains selected as representative isolates for further analyses as categorised according to molecular typing data and statistical associations.

Isolate name	PFGE	Clinical data	Source	Status	MLST ST	MLST CC	*spa*-CC	SCC*mec*	*agr*	PVL	Statistical association
THW38	THW-A	Lip abscess	SSTI	Major	1865	30	21	NA	III	+	MSSA/PVL+/SSTI
THW382	THW-B	Laparotomy (day 15 post-operative)	SSTI	Inter	36	30	21	II	III	-	MRSA/PVL-/Adults/SSTI
THW146	THW-C	Abscess left knee	SSTI	Major	22	22	891	NA	I	+	MSSA/PVL+/SSTI
THW393	THW-C	Septic left tibia ex-fix	BJ	Major	22	22	891	NA	I	+	HIV+ (isol.)
THW366	THW-E	Chronic folliculitis	SSTI	Major	121	121	NF:16	NA	IV	+	MSSA/PVL+/SSTI
THW262	THW-O	Pyrexia, intracostal drainage site	RT	Major	612	8	64	IV	I	-	MRSA/PVL-
THW65	THW-G	Septic ORIF	BJ	Inter	1862	8	1597	NA	I	-	MSSA/Adults
THW271	THW-J	Sinus fungal infection	ENT	Inter	8	8	64	V	I	-	PVL-/Adults
THW81	THW-L	Sepsis, pin tract	PROST	Inter	239	8	21	III	I	-	MRSA/PVL-
THW70	THW-N	Sepsis, nosocomial MRSA	SSTI	Inter	612	8	64	IV	I	-	MRSA/PVL-
THW195	THW-K	Impetigo	SSTI	Minor	8	8	64	NA	I	-	HIV+ (ass.)
THW273	THW-P	Pus from hand	SSTI	Minor	612	8	64	IV	I	-	HIV+ (ass.)
THW93	THW-O	Sepsis	UK	Major	612	8	64	IV	I	-	HIV+ (isol.)
THW99	THW-L	Septic wound	SSTI	Inter	239	8	21	UK	I	-	Non-typeable SCC*mec*
THW122	THW-S	Sepsis	SSTI	Major	15	15	84	NA	II	-	MSSA/PVL-
THW412	THW-V2	No clinical data supplied	UT	Major	1	15	NF:14	NA	III	-	MSSA
THW224	THX-X	Wound sepsis	SSTI	Inter	188	15	EX	NA	I	-	MSSA/PVL-

Table B1 Strains selected as representative isolates for further analyses as categorised according to molecular typing data and statistical associations.

Isolate name	PFGE	Clinical data	Source	Status	MLST ST	MLST CC	*spa*-CC	SCC*mec*	*agr*	PVL	Statistical association
THW17	THW-Y	Wound sepsis	SSTI	Major	1863	5	2	NA	II	-	MSSA/PVL-
THW235	THW-Z	Measles	EY	Inter	1864	5	2	NA	II	+	MSSA
THW264	THW-AA	Conjunctivitis	EY	Inter	5	5	2	I	II	-	MRSA/PVL-
THW64	THW-V1	Suspect TB, pericardial fluid	CA	Major	6	6	64	NA	I	-	MSSA/PVL-
THW356	THW-EE	Hip abscess	SSTI	Major	45	45	EX	NA	I	-	MSSA/PVL-
THW368	THW-V3	Wound infection	SSTI	Inter	12	12	888	NA	II	+	MSSA/PVL+
THW241	THW-W	ESRF	IVD	Inter	97	97	267	NA	I	-	MSSA/PVL-/Males
THW255	THW-BB	Infected leg	SSTI	Inter	88	88	186	IV	III	+	PVL+/SSTI

ass. = associated; isol. = isolated; ESFR = end-stage renal failure; ORIF = open reduction and internal fixation; Inter = intermediate; NA = not applicable; Ex = excluded; SSTI = skin and soft tissue; BJ = bone and joint; RT = respiratory tract; ENT = ear, nose and throat; PROST = prosthetic device; UK = unknown; UT = urinary tract; EY = eye; CA = cardiac; IVD = intravascular device.

CHAPTER 4: Host cellular invasiveness and host cell death induction of representative clinical *S. aureus* isolates

4.1 Introduction

4.1.1 Pathogenesis of infection:

As previously stated, *S. aureus* can establish an infection after adherence and invasion of host cells. *S. aureus* can also disseminate from the blood into the surrounding tissue[269]. The host cellular integrins $\alpha_5\beta_1$ are utilised as the host cellular receptor for invasion[102, 103], accessed by the bacterial protein FnbA/B or Eap[103, 104]. After invasion, the organism has to escape from the phagolysosome, or be killed. If phagosomal escape is successful, the organism can replicate or persist inside the host cell or even kill the cell[83, 270] usually through the production of toxins. However, no clear biological concept currently exists for *S. aureus* and the biological role of phagosomal escape is also not clear. It is also known that different cell types may react in different manners to infection by *S. aureus*[271].

4.1.2 Host cellular invasins:

4.1.2.1 Fibronectin-binding proteins (FnbPs):

Sinha *et al.*, amongst other, have clearly demonstrated the role of FnbPs as the main invasin of *S. aureus* through a series of loss/gain of function experiments and identified a fibronectin-dependent bridging mechanism to the host cellular integrin $\alpha_5\beta_1$[103, 233] for epithelial, endothelial and fibroblast cells. FnbPs do not require any other *S. aureus*-specific co-receptors to confer invasiveness and this function can be accomplished by either FnbA or FnbB[104] which must be anchored into the

bacterial cell wall, as truncation of these proteins results in deficient adherence and cellular invasiveness[89]. This is illustrated in Figure 4.1.

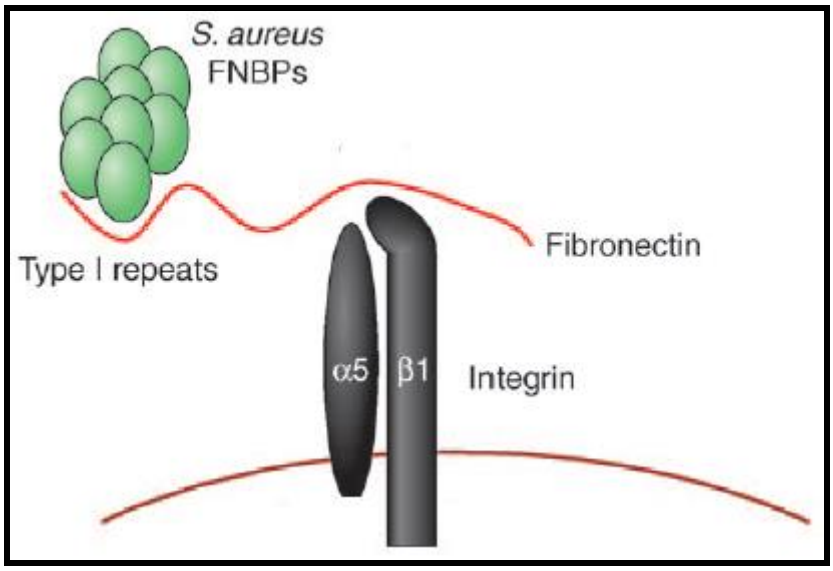


Figure 4.1 Schematic representation of the co-localisation of the FnbPs-fibronectin bridging complex and the human host cellular receptor, $\alpha_5\beta_1$. Adapted from Hauck *et al.*[102].

4.1.2.2 Extracellular adherence protein (Eap):

It has been shown that the protein Eap, with its broad binding capacity, can play a role in the cellular invasion of host cells[254, 272], such as fibroblasts and HACAT (keratinocytes) cells and also inhibits the recruitment of host leukocytes[252], although it has many other functions, incl. matrix binding. Eap can very easily be used as an invasin in genetic backgrounds where FnbPs are not produced or defective, such as for strain Newman[272], whose FnbPs are mutated and secreted. The molecule ICAM-1 (endothelial cell adhesion molecule) has been identified as a major host cellular receptor for Eap[251] during the inhibition of leukocyte recruitment. In order for Eap-based cellular invasion of *S. aureus* to occur, more than one tandem repeat domain of the Eap protein is required[88].

4.1.2.3 Plasmin sensitive surface protein (Pls):

Pls, an Sdr-family protein, has so far only been associated with SCC*mec* type I clinical MRSA isolates[37, 273] and significantly reduces the ability of these isolates to adhere to several mammalian proteins, including fibronectin and fibrinogen[273]. Expression of this protein also reduces the cellular invasiveness of the MRSA isolates[257]. The invasiveness of MRSA can be predicted using three properties, as shown by Werbick *et al.*: (1) expression of *pls*; (2) SCC*mec* type; and (3) *spa* type[259]. Pls acts by steric hindrance and affects the adherence and cellular invasiveness of an isolate irrespective of the MRSA/SCC*mec* background[256].

4.1.3 Phagosomal escape of internalised staphylococci:

Both the induction of host cell death[274, 275] or intracellular persistence[276] can result after successful phagolysosomal escape by *S. aureus*. However, only a small number of clinical and laboratory isolates are capable of phagolysosomal membrane destruction, as the maintenance of phagolysosomal integrity after invasion of non-professional phagocytes has been displayed[277]. α-toxin, a pore forming toxin, has been proposed to be involved in the escape of the organism from the phagolysosome[270], which is thought to be a multifactorial process. Following escape, the micro-organism can either be killed by host cells, or it can persist and cause recurrent and/or persistent infections[83]. Conflicting data is currently available regarding which virulence factor(s) is/are involved in the process of phagosomal escape. α-toxin has been identified as a requirement for vesicular escape in a cystic fibrosis epithelial cell line, CFT-1[278], and in CHO cells (ovary hamster cells)[279]. However, in upper-airway epithelial cells, α-toxin is not sufficient to allow for the phagosomal escape of *S. aureus*[280]. Giese *et al.* clearly demonstrated the synergistic actions of both α- and β-toxin in phagosomal escape in human epithelial and endothelial cells[281].

4.1.4 Intracellular persistence of *S. aureus*:

S. aureus can survive inside human leukocytes, such as macrophages and non-professional phagocytes[270], even though the majority of internalised staphylococci will be killed. Inside these cells, the organism can (1) evade the host immune system and antimicrobials; (2) use mobile cells

to disseminate throughout the host and then kill these cells; or (3) kill the cells immediately. Macrophages and neutrophils infected by *S. aureus* display increased expression of anti-apoptotic genes, thus allowing bacterial intracellular survival[101, 282, 283], while their viability and mobility is initially not affected[284], and has been shown to play a very important role specifically during CA-MRSA infection[285]. Although multiple virulence factors determine the survival of *S. aureus* after phagocytosis, α-toxin seems to be a key component[284].

It is thought that persistence inside non-professional phagocytes can result in recurrent infections, such as recurrent tonsillitis[286]. Bacterial isolates with certain genotypic and phenotypic characteristics have been associated with persistent infection. Xiong *et al.* identified SCC*mec* II, CC30 and *spa* t016 to be associated with persistent bacteremia[287] in their setting.

4.1.5 Induction of host cell death:

In any given *S. aureus* strain, host cell death induction is difficult to predict and depends on many factors[288], including bacterial genetic background, host cell type and pathway of cell death induction to be used. Various bacterial virulence factors are also involved, of which α-toxin[289, 290] and PVL are most prominent.

Intracellular *S. aureus*, if viable, can exist free in the cytoplasm and kill endothelial cells, partly by apoptosis, as shown by nuclear morphology and DNA fragmentation[275]. Metabolically active intracellular staphylococci are required for the induction of apoptosis in endothelial cells, which is dependent on *agr* and *sigB*[7]. Strains with invasive and haemolytic phenotypes are normally associated with caspase-dependent induction of apoptosis, while non-invasive haemolytic and non-haemolytic invasive isolates are not[7]. For the induction of apoptosis in pulmonary epithelial cells, bacterial internalisation, as well as bacterial intracellular replication is required[274]. This is not the case for all epithelial cell lines.

In mesothelial cells, invasive *S. aureus* strains producing α-toxin are associated with the ability to induce host cell death. Host cell death is induced via apoptosis, independently of caspases[291]. *S. aureus* biofilms are also able to induce apoptosis in human keratinocytes[292]. *S. aureus* released

from dead or dying cells are capable of re-infecting new cells, thus continuing the progress of infection by establishing persistent or recurrent infections, as in the case of osteomyelitis.[293].

4.1.5.1 α-toxin:

S. aureus α-toxin triggers the activation of caspase and DNA fragmentation in Jurkat T-cells via the intrinsic death pathways, irrespective of the cell death receptor[294], resulting in apoptosis of the host cell. However, this toxin can also lead to the destruction of its host cell in a caspase-independent manner even if caspases are activated, resembling cell necrosis[295] which is characterised by cell swelling and cytoplasmic vacuolation. In Jurkat T-cells, α-toxin induced apoptosis is exclusively facilitated by the mitochondrial pathway, whereas in human peripheral blood mononuclear cells (MNC), endogenous tumour necrosis factor-α (TNF-α) and a death receptor-dependant pathway are involved[296]. This toxin has also been identified as a prominent factor for causing *S. aureus*-based pneumonia and necrotic disease[297]. In monocyte-derived cells, α-toxin activates the NLRP3-inflammasome (nucleotide-binding domain and Leucine-rich repeats containing gene family, pyrin domain containing 3) which results in the activation of caspase-1, leading to cell necrosis[298]. This is also the case *in vivo*[299]. Low doses of α-toxin usually lead to apoptosis, whereas high doses lead to cell necrosis[296].

4.1.5.2 Panton-Valentine leukocidin (PVL):

The two component toxin PVL, another pore-forming toxin, targets PMNs[91], is encoded by the *lukS/F* genes and is associated with distinct lineages of CA-MRSA[67]and some endemic MSSA lineages. It is one of the main virulence factors required to cause necrotising pneumonia[66, 92] or skin-associated infections[65]. However, the role of PVL as a major virulence factor for CA-MRSA is questioned, as some studies have shown that PVL- strains are as virulent as their PVL+ counterparts[95]. This toxin also displays species and cell-type dependant cytotoxicity[91].

4.1.5.3 Other toxins:

The hemolysin β-toxin, a sphingomyelinase C, induces lysis of erythrocytes depending on the amount of sphingomyelin present in the plasma membrane[300]. This toxin can also kill monocytes, an action associated with cytokine-related events[300]. δ-toxin is a pore-forming toxin (PFT) which has haemolytic activity and can activate neutrophils *in vitro*[101] and is structurally related to PSMs. γ-toxin is one of the most important and prominent leucotoxins produced by *S. aureus* and is secreted by 99% of all strains[101]. PSMs are highly effective leucotoxins (also PFTs) which were discovered not too long ago. They seem to play a very important role in the pathogenesis of MSSA and CA-MRSA infections, especially during necrotising disease and *in vivo* during murine skin abscess and bacteraemia models[49]. Other leukocidins, such as LukGH and LukAB, can also be expressed by some CA-MRSA, such as USA300 isolates[301, 302]. These toxins also have a cytotoxic effect on human PMNs and can work in synergism with PVL to enhance virulence.

4.1.6 Regulation of host cellular invasion and host cell death induction:

Both the global regulators *agr* and *sar* are involved in invasion and induction of host cells[78]. *Agr*$^-$, *sar*$^-$ and *agr*$^-$*sar*$^-$ mutants of a laboratory isolate were able to invade mammalian cells at much greater levels than their parent strain, but all failed to induce apoptosis[78]. *Agr* deficient isolates fail to express *agr*-dependent virulence factors, such as α-toxin and struggle to induce cell death[303]. USA300 CA-MRSA isolates activate their *agr* operon in human polymorphonuclear cells (PMNs) resulting in PMN lysis by α-toxin[304]. Both of these regulators are also activated upon *in vivo* infection[305]. The organism adapts after internalisation by up-regulating genes involved in virulence[306] and iron-scavenging and down-regulating those involved in cell division and nutrient transport[276]. Differences exist between the regulation of virulence factors, such as α-toxin, *in vivo* when compared to *in vitro* conditions[307]. *In vivo*, the regulator *sae* plays an important role, which, together with *agr* have been identified as independent up-regulators of virulence[308].

Other regulators have also been identified, such as *rsr* (repressor of *sarR*), that represses the *agr* locus, thus inhibiting virulence[309].

4.2 Aims and Objectives

4.2.1 Study aim:

The aim of the research presented in this chapter was to investigate the cellular invasiveness and host cell death induction of representative *S. aureus* isolates. The representative isolates were selected from various clonal lineages previously identified to be in circulation and are associated with clinical or bacterial characteristics, such as methicillin-resistance, male gender or patient HIV+ status. The same representative isolates as in the previous chapters were used.

4.2.2 Study objectives:

1. To investigate the abilities of the representative isolates to invade human cells using a flow cytometry-based assay.
2. To investigate the abilities of the representative isolates to induce the death of human cells by measuring the following three end points:
    a. Hypodiploid nuclei as an indicator of the induction of apoptosis;
    b. LDH release as an indicator of cell necrosis; and
    c. Respiratory activity of mitochondria as an early indicator of cell viability.
3. To investigate if any statistical associations are present between invasiveness or cell death induction and:
    a. Clonality;
    b. Methicillin-resistance;
    c. Bacterial PVL status, i.e. PVL+ or PVL-; and
    d. Patient HIV status.

4.3 Materials and Methods

4.3.1 Chemicals, reagents, media and kits:

All chemicals, reagents, consumables and kits used during this research were analytical and/or molecular biology grade.

4.3.2 Bacterial strains and growth conditions:

All bacterial isolates used were sub-cultured on 5% blood agar plates (Bio-Merieux, France) and incubated overnight at 37 ºC. 2-3 colonies of bacteria were used to inoculate 20 ml Mueller-Hinton broth (MHB), which was incubated statically for 16 h at 37 °C.

4.3.3 Control strains and compounds:

The laboratory strain Cowan I was used as an invasive control (positive control) for the flow cytometric-based invasion assay. The *S. carnosus* strain TM300 was used as a non-invasive control (negative control) for the invasion assay and as a non-cytotoxic control (negative control) for all three of the cell death assays employed. The laboratory isolate 6850 was used as a cytotoxic control (positive control) for all three cell death assays. During all cell death assays, Triton-X (Roth, Germany) and staurosporine (Roche Diagnostics, Germany) were also included as cytotoxic chemical controls (positive controls). Staurosporine induces cell death through the inhibition of protein kinase C, while Triton-X, a detergent, is known to be a potent cytotoxic compound. All buffer compositions are listed in Tables 4.1 and 4.2 at the end of this section.

4.3.4 Flow cytometric invasion assay:

The cellular invasiveness of the representative isolates was determined by adapting a previously published flow cytometric-based invasion assay[103, 310]. After the labelling of the bacterial cultures with fluorescein isothiocyanate (FITC) (Fluka BioChemika, Switzerland), they were fixed and

allowed to invade cultured mammalian cells. Following this, the fluorescent signals emitted from the trypsinised wells were measured using a FACS Calibur (BD Biosciences, USA) and compared to that of an invasive control (set at 100%) and a non-invasive isolate (<10% of invasive control).

4.3.4.1 FITC-labelling and fixation of bacteria:

Bacteria were grown in 50 ml tubes as described in section 4.3.2. The tubes were centrifuged at 4 000 rpm for 5 min, the supernatant discarded and the bacterial pellet was completely dissolved in 2.5 ml 1× PBS. 2.5 ml 4% formaldehyde (Roth, Germany) was added to the bacterial suspension and mixed thoroughly by vortexing. The bacterial suspension was incubated for 1 h at 37 °C to allow for bacterial fixation, where after the tubes were centrifuged again at 4 000 rpm for 5 min, the supernatant discarded and the pellet washed thoroughly with 5 ml 1× PBS by vortexing.

Next, the FITC-staining solution was prepared as follows: a 4.2% $NaHCO_3$ (Roth, Germany) and a 5.3% Na_2CO_3 (Roth, Germany) solution were prepared and 5.8 ml of the 4.2% $NaHCO_3$ solution was mixed with 10 ml of the 5.3% Na_2CO_3 solution, thus preparing the FITC-buffer. 1 mg FITC powder was dissolved in 1 ml DMSO (Invitrogen, USA), where after 9 ml of the FITC-buffer was mixed with the 1 ml FITC-DMSO solution, thus preparing the FITC-staining solution.

Following this, the freshly washed bacterial suspension was centrifuged again at 4 000 rpm for 5 min and the pellet completely dissolved in 3 ml FITC-staining solution. The bacterial-FITC suspension was then incubated at 37 °C for 30 min, centrifuged at 4 000 rpm for 5 min and the pellet washed with 1× PBS. The FITC-labelled bacterial culture was then standardised to OD 1_{540nm} as described in Chapter 3, section 3.3.5.

A fresh overnight culture was fixed, labelled and normalised for every experiment.

4.3.4.2 Mammalian cell culture:

293 cells (adenovirus type 5 DNA-transformed primary human embryonic kidney cells), an adherent cell line, were used to investigate the cellular invasiveness of the representative isolates. (www.atcc.org) 293 cells were maintained in DMEM/F-12 (Invitrogen, USA) supplemented with

10 % FCS (PAA Laboratories, Austria) and 1× Pen/strep mix (100 U/ml Penicillin and 100 µg/ml Streptomycin) (Cambrex Bio Science, Belgium). A medium-sized flask was split twice weekly 1:5 by 1 ml of 1× Trypsin-EDTA (Invitrogen, USA). Cells were only used up to passage 25 and maintained in humidified air (37°C with 5% CO_2).

4.3.4.3 Invasion assay:

293 cells (epithelial cells) were plated in 24-well culture dishes at 300 000 cells/well on the day before the assay. On the day of the assay, the cells were washed once with 500 µl invasion medium. 500 µl invasion medium was added to every well and each plate was pre-cooled at 4°C for 20 min. Following this, 50 µl bacterial suspension (adjusted to OD 1_{540nm}) was added to every well, resulting in a MOI (multiplicity of infection) ~ 30 and incubated for 1 h at 4°C to allow for bacterial sedimentation onto the cell monolayer and to synchronise the following internalisation step. The remaining bacterial suspension was kept in the dark at 4°C for later FLOW-analyses. After the initial 1 h incubation, the cells were moved to 37 °C with 5% CO_2 for 3 h to allow for bacterial invasion. After invasion, the cells were removed from the incubator, washed once with 1× PBS and trypsinised with 200 µl Trypsin-EDTA (Invitrogen, USA). 1 ml stop medium was quickly added to each well to stop trypsinisation and the cells were resuspended and collected in FACS tubes.

The following controls were included: strains Cowan I and TM300, as mentioned in section 4.3.3, and cells only (without bacteria).

4.3.4.4 Flow cytometry:

After collection of the cells in the FACS tubes, the tubes were centrifuged at 1 000 rpm for 5 min at 4 °C and the supernatant aspirated with an aspiration pump. The cell pellet was resuspended in 500 µl of 1% HSA/PBS, 1 µl/ml monensin (25 mM) (Fluka BioChemika, Switzerland) was added to each tube (to neutralise fluorescence quenching) and mixed carefully to not disrupt the cells. The tubes were incubated in the water bath at 37 °C for 15 min and placed on ice. Before measurement

of the cells, 50 µl propidium iodide (PI) (5 µg/ml) (Fluka BioChemika, Switzerland) was added to each tube and vortexed to allow for PI-based exclusion of dead cells.

Cells were analysed on a FACS Calibur (BD, New Jersey, USA), with which a pre-set fixed forward and side scatter (FSC/SSC) gating and fixed amplification was used for the 293 cells. Fluorescence was analysed in the Fl-1 channel for FITC and Fl-3 channel for PI. After acquisition, histograms of the fluorescence (Fl-1 height) of FSC/SSC-gated cells were obtained. Less than 2% of the cells of the respective cells-only control were included in the fluorescence intensity marker (M), which was pre-set.

The FITC-labelled bacterial suspension was also analysed with the FACS Calibur to determine the mean fluorescence intensity of the bacterial population, which was measured also in the Fl-1 channel.

4.3.4.5 Analyses of flow cytometric data:

The results obtained for the cells were normalised according to the mean fluorescence intensity of the respective bacterial population. The results obtained were expressed as the average of the mean values with the SEM (standard error of the mean) of three independent experiments performed in duplicate. Cellular invasiveness of the representative isolates was expressed as a percentage relative to the positive control (Cowan I), which was set at 100%. An arbitrary cut-off value of 50% (relative to the invasive control Cowan I) was used to discriminate between invasive (≥ 50%) and non-invasive (< 50%) isolates.

4.3.5 Induction of cell death assays:

The abilities of the representative isolates to induce the death of host-cells were investigated using 3 different end points, as no data regarding the potential cytotoxicity of South African *S. aureus* isolates is available and it is known that *S. aureus* can induce host cell death using a variety of pathways, i.e. apoptotic and necrotic. The Nicoletti assay is a flow cytometric assay that measures the proportion of hypodiploid nuclei (an indicator of the induction of apoptosis) present in a population of cells. The 2nd assay used to measure cell death was a cytotoxicity detection kit, where

the release of the enzyme lactate dehydrogenase (LDH) into the culture supernatant was measured, normally a signal of cell necrosis. The last assay used was a cell-viability assay using the reagent WST-1, which is cleaved to formazan by the mitochondrial dehydrogenases of metabolically active cells.

4.3.5.1 Nicoletti assay:

An adapted version of this previously published protocol was used[7, 311]. Bacteria were grown in 50 ml tubes as described in section 4.3.2 and standardised to OD 1_{540nm} as described in Chapter 3, section 3.3.5. A fresh overnight culture was standardised for every experiment.

Ea.hy926 (endothelial cells)[312] (human umbilical vein cell line) (www.atcc.org), an adherent cell line, were used to investigate the abilities of the representative isolates to induce cell death. Ea.hy926 cells were maintained in DMEM/F-12 (Invitrogen, USA) supplemented with 10% FCS (Invitrogen, USA) and 1× Pen/strep mix (100U/ml Penicillin and 100 µg/ml Streptomycin) (Cambrex Bio Science, Belgium). A medium-sized flask was split twice weekly 1:4 by 1× Trypsin-EDTA (Invitrogen, USA). Cells were only used up to passage 25 and maintained in humidified air (37°C with 5% CO_2).

Ea.hy926 cells were plated in 24-well culture dishes at 300 000 cells/well on the day before the assay. On the day of the assay, the cells were washed once with 500 µl invasion medium, where after 500 µl invasion medium was added to every well and each plate was pre-cooled at 4 °C for 30 min. Following this, 50 µl bacterial suspension (adjusted to OD 1_{540nm}) was added to every well, resulting in a MOI ~ 30 and incubated for 1h at 4 °C to allow for bacterial sedimentation. After the initial 1 h incubation, the cells were moved to 37 °C with 5% CO_2 for 3 h to allow for bacterial invasion. After invasion, the cells were removed from the incubator and washed once with 1× PBS. Fresh, full DMEM/F-12 (Invitrogen, USA) media, supplemented with lysostaphin (20 µg/ml) (AMBI, USA) was added to every well and the cells were incubated for a further 21 h at 37 °C with 5% CO_2. After this, the supernatant of each well was transferred to a FACS tube, the cells washed once with 500 µl 1× PBS (which was also transferred to its respective FACS tube) and then trypsinised with 200 µl Trypsin-EDTA (Invitrogen, USA). The 24-well plates were shaken slightly to dislodge all cells1 ml, stop medium was added to each well to stop trypsinisation, cells were

resuspended and transferred to the respective FACS tubes for flow cytometric analyses. The following controls were included: strains 6850 and TM300, as mentioned in section 4.3.3, and cells only (without bacteria). The following chemical controls, known to induce cell death, were also included: 1% Triton-X, 1 µM and 0.1 µM staurosporine (Roche Diagnostics, Germany).

After collection of the cells in the tubes, the tubes were centrifuged at 4 000rpm for 5 min at 4 °C and the supernatant aspirated with an aspiration pump until 100 µl was left, which was used to resuspend the cell pellet with. 500 µl Nicoletti-PI buffer was added to every FACS tube, which was vortexed immediately to keep the bacterial pellet resuspended. The cells were incubated in the dark for 1 h at 4 °C and analysed on ice using a FACSCalibur (BD, New Jersey, USA) at 488 nm laser excitation.

Cells were analysed on a FACSCalibur (BD, New Jersey, USA), with which a pre-set fixed forward and side scatter (FSC/SSC) gating and fixed amplification was used for the Ea.hy926. Fluorescence was analysed in the Fl-2 channel for PI. After acquisition, histograms of the fluorescence (Fl-2 height) of FSC/SSC-gated cells were obtained. Less than 3% of the cells of the respective cells-only control were included in the fluorescence intensity marker (M), which was pre-set. The PI fluorescence peak from the nuclei of the viable cells-only control was set at channel >1000. Nuclei from apoptosis cells should give a PI fluorescence peak in channels 5-1000, i.e. below that of the viable cells.

The results obtained were expressed as the average of the mean values with the SEM of three independent experiments performed in duplicate. The percentage of the number of intact cells present, relative to the cells only control which was set at 100%, was determined as x, and the equation $1 - x$ was used to determine induction of host cell death of the representative isolates, i.e. the % of dead cells. An arbitrary cut-off value of 50% (relative to the cytotoxic control) was used to discriminate between cytotoxic ($\geq$ 50%) and non/moderately cytotoxic ($<$ 50%) isolates.

4.3.5.2 LDH assay:

This assay was performed using the "LDH cytotoxicity" kit (Roche Diagnostics, Germany) as per the recommendation of the manufacturer. Bacteria were grown as in 50 ml tubes as described in section 4.3.2 and standardised to OD 1_{540nm} as described in Chapter 3, section 3.3.5. A fresh overnight culture was standardised for every experiment.

Ea.hy926 cells were maintained as described in Section 4.3.5.1. Ea.hy926 cells were plated in 96-well culture dishes at 50 000 cells/well on the day before the assay. On the day of the assay, the cells were washed once with 500 µl invasion medium, where after 500µl invasion medium was added to every well and each plate was pre-cooled at 4 °C for 30 min. Following this, 20 µl bacterial suspension (adjusted to OD 1_{540nm}) was added to every well, resulting in a MOI ~ 30 and incubated for 1 h at 4 °C to allow for bacterial sedimentation. After the initial 1 h incubation, the cells were moved to 37 °C with 5% CO_2 for 3 h to allow for bacterial invasion. After invasion, the cells were removed from the incubator and washed once with 1× PBS. Fresh, full DMEM/F-12 media (Invitrogen, USA), supplemented with lysostaphin (20 µg/ml) was added to every well and the cells were incubated for a further 21 h at 37 °C with 5% CO_2. 1 h before the end of the 21 h incubation period, Triton-X (1%) was added to its respective wells and the cells were incubated for the remaining hour. After this, the 96-well plate was centrifuged for 10 min at 1 000 rpm, where after 25 µl of the supernatant from each well was transferred to the same well in a fresh, sterile 96-well plate, already containing 150 µl 1× PBS/well and the original plate was stored at 4 °C in the dark. The LDH reaction mixture was prepared fresh every time as per the manufacturers´ recommendation and 25 µl of this mixture was added to every well of the plate containing the supernatant-PBS mixture. The plate was then incubated at RT for 30 min, covered in aluminium foil, where after the absorbance of each well was measured using an ELISA reader.

The following controls were included: strains 6850 and TM300, as mentioned in section 4.3.3, cells only (without bacteria) and media only (without cells and bacteria). The following chemical controls, known to induce cell death, were also included: 1% Triton-X, 10 µM and 1 µM staurosporine.

The absorbance was measured using a Tecan Reader Infinite F200 Pro (Tecan GmbH, Germany) at 490nm, with a reference reading at >600nm. The value obtained from the media only control was subtracted from the values obtained for all the remaining controls and all strains tested. % cytotoxicity was determined using the following equation:

$$\%\ \text{cytotoxicity} = \frac{\text{experimental value} - \text{low control}}{\text{high control} - \text{low control}} \times 100$$

The value obtained for the cells only control was used as the low control and those obtained for the 1% Triton-X was used as the high control. Using the above given formula, when the low control value is used as the "experimental value", the value for the cells only control (negative control) is set to 0% cytotoxicity, and when the high control value is used, the value for the 1% Triton-X is set to 100% cytotoxicity.

The results obtained were expressed as the average of the mean values with the SEM of three independent experiments each performed in triplicate. The abilities of the representative isolates to induce cell death was measured by the release of LDH from the cytosol into the culture supernatants and were expressed as a percentage relative to the 1% Triton-X control, which was set at 100%. The % cytotoxicity for strain 6850 was expected to be >70% and that of strain TM300 <10%. An arbitrary cut-off value of 50% (relative to the cytotoxic control) was used to discriminate between cytotoxic (≥ 50%) and non/moderately cytotoxic (< 50%) isolates.

4.3.5.3 WST-1 cell viability assay:

This assay was performed using the WST-1 ready-to-use reagent[296] (Roche Diagnostics, Germany) as per the recommendation of the manufacturer. This assay was designed in a 96-well plate format for the spectrophotometric quantification of cell viability. WST-1, a tetrazolium salt, is cleaved to formazan by the mitochondrial dehydrogenases of metabolically active cells. An ELISA reader can then be used to measure the absorbance of the sample, thus quantifying the formazan dye.

Bacteria were grown as in 50 ml tubes as described in section 4.3.2 and standardised to OD 1_{540nm} as described in Chapter 3, section 3.3.5. A fresh overnight culture was standardised for every experiment.

Ea.hy926 cells were maintained as described in section 4.3.5.1. Ea.hy926 cells were plated in 96-well culture dishes at 50 000 cells/well on the day before the assay. On the day of the assay, the cells were washed once with 500 µl invasion medium, where after 500 µl invasion medium was added to every well and each plate was pre-cooled at 4 °C for 30 min. Following this, 20 µl bacterial suspension (adjusted to OD 1_{540nm}) was added to every well, resulting in a MOI ~ 30 and incubated for 1 h at 4 °C to allow for bacterial sedimentation. After the initial 1 h incubation, the cells were moved to 37 °C with 5% CO_2 for 3 h to allow for bacterial invasion. After invasion, the cells were removed from the incubator and washed once with 1× PBS. Fresh, full DMEM/F-12 (Invitrogen, USA) media, supplemented with lysostaphin (20 µg/ml) was added to every well and the cells were incubated for a further 21 h at 37 °C with 5% CO_2. Following this incubation step, 5 µl WST-1 reagent (Roche Diagnostics, Germany) was added to every well and mixed by orbital agitation for 10 s on an orbital shaker at 100 rpm. The cells were incubated for another 2 h at 37 °C with 5% CO_2, where after the absorbance of each well was measured using an ELISA reader.

The following controls were included: strains 6850 and TM300, as mentioned in section 4.3.3, cells only (without bacteria) and media only (without cells and bacteria). The following chemical controls, known to induce cell death, were also included: 1% Triton-X, 1 µM and 0.1 µM staurosporine.

The absorbance was measured using a Tecan Reader Infinite 200 Pro (Tecan GmbH, Germany) at 490nm, with a reference reading at >600nm. The value obtained from the media only control was subtracted from the values obtained for all the remaining controls and all strains tested.

The results obtained were expressed as the mean of the mean with the SEM of three independent experiments performed in triplicate. The abilities of the representative isolates to induce cell death were determined after the % of viable cells relative to the cells only control, which was set at 100%, was deducted from 1 and expressed as a %. An arbitrary cut-off value of 50% (relative to the cytotoxic control) was used to discriminate between cytotoxic (≤ 50%) and non/moderately cytotoxic (> 50%) isolates.

4.3.6 Statistical associations:

4.3.6.1 Associations between invasion or host cell death induction and methicillin-resistance or clonality:

To investigate if any associations existed between invasion/cell death and methicillin-resistance, two tests were performed. Firstly, as a parametric comparison, the 2-sample t-test was also used. A confidence interval of 95% was used and an association was regarded as statistically significant if a p-value < 0.05 was obtained. Secondly, as a non-parametric comparison, the 2-sample Wilcoxon rank-sum test (Mann-Whitney test) was used. An association was regarded as statistically significant if a p-value < 0.05 was obtained.

To investigate if any associations existed between invasion/cell death and clonality, the Kruskal-Wallis test was performed as a comparison using non-parametric inference. In order for us to compare all groups in a valid fashion, a group was created which was composed of all the singletons. An association was regarded as statistically significant if a p-value < 0.05 was obtained.

4.3.6.2 Associations between invasion or host cell death induction and patient HIV status or bacterial PVL status:

To investigate if any associations existed between invasion or host cell death induction and patient HIV status, the Kruskal-Wallis test was performed as a comparison using non-parametric inference. An association was regarded as statistically significant if a p-value < 0.05 was obtained.

To investigate if any associations existed between invasion or host cell death induction and bacterial PVL status, i.e. PVL-positive or negative, the 2-sample Wilcoxon rank-sum test (Mann-Whitney test) was used. An association was regarded as statistically significant if a p-value < 0.05 was obtained.

4.3.7 Composition of agar, broth, media and buffers:

Table 4.1 Composition of agar and broth used during invasion and cell death assays.

Media	Composition
5% Blood agar plate	15 g pancreatic digest of casein; 15 g papaic digest of soy meal; 5 g NaCl; 15 g agar; Bring to volume of 1l and autoclave Adjust pH to 7.3 Cool to 45-50°C; add 5% (v/v) sterile defibrinated blood; mix
MH broth	2 g dehydrated beef infusion; 17.5 g casein hydrolysate; 1.5 g starch; Bring to volume of 1l and autoclave Adjust pH to 7.4

Table 4.2 Composition of media and buffers used during invasion and cell death assays.

Buffer/Solution	Composition
1× PBS	8 g NaCl; 0.2 g KCl; 1.44 g Na_2HPO_4; 0.24 g KH_2PO_4 in 800 ml dH_2O Adjust pH to 7.4 Add H_2O to 1 l and autoclave
1% HSA/PBS	1:20 of 20% HSA in PBS
Invasion medium	1%HSA; 10 mM HEPES; white DMEM/F12
Stop medium	10% FCS in white DMEM/F12
Nicoletti-PI buffer	0.1% Triton-X; 0.1% sodium citrate; 50 µg/ml PI

4.4 Results

This chapter deals with the cellular invasiveness and capabilities to induce host cell death of the set of representative *S. aureus* clinical isolates. We determined invasiveness in 293 cells using a FACS-based assay and the ability to induce cell death using 3 assays for three different end points: (1) A FACS-based assay measuring hypodiploid nuclei as a sign of apoptosis; (2) the amount of LDH released into the supernatant as a sign of cell necrosis; and (3) the chemical conversion of the reagent WST-1 as a sign of cell viability.

4.4.1 Cellular invasiveness:

The cellular invasiveness of the representative isolates was determined using a FACS-based assay with FITC stained bacteria, which included bacterial invasive and non-invasive controls. These controls behaved as expected.

As can be seen in Figure 4.2, the cellular invasiveness of the 25 representative isolates were quite diverse (range 42.4% - 211.6% relative to the invasive control [Cowan I set at 100%]; mean = 142.6%; median = 142.2%). Using an arbitrary cut-off value of 50% to distinguish non-invasive from invasive isolates, all isolates, irrespective of the MLST CC were invasive, except for the ST121 isolate, THW-366 (MSSA PVL+, associated with SSTI). Two isolates could be classified as highly invasive (>200%): THW-273 (ST612:CC8; HIV+ clone) and THW-356 (ST45:CC45; PVL-MSSA). Due to the inability of the bacterial pellet to dissolve in PBS after fixations, we were unable to determine the invasiveness of isolate THW-264 for repeated experiments (ST5:CC5; SCC*mec* I; PVL- MRSA).

No significant difference in invasiveness was observed between isolates from HIV+ or HIV- patients (Figure 4.3).

The invasiveness of the non-invasive control, TM300, was <10% relative to Cowan I.

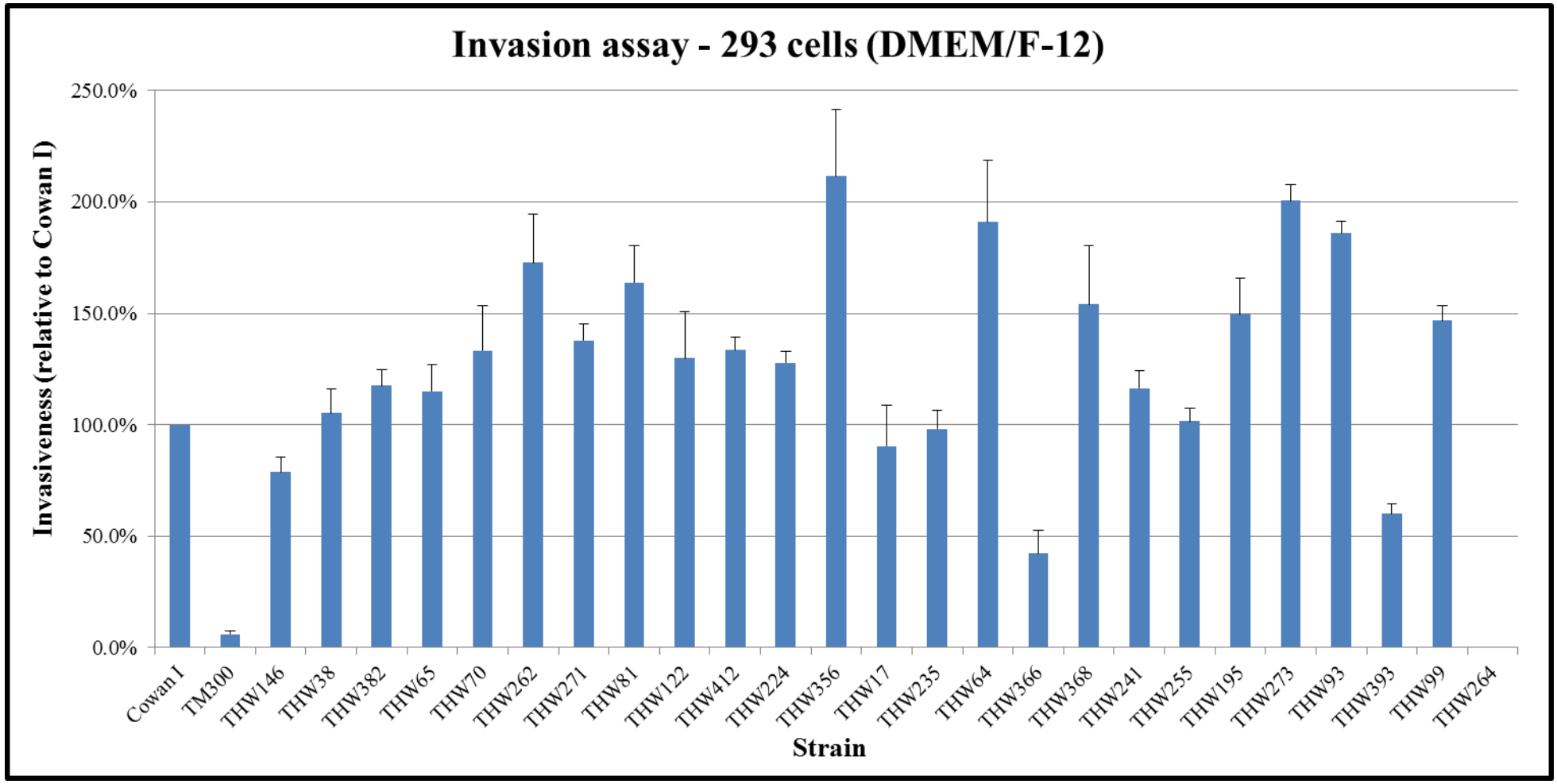


Figure 4.2 Differences in host cell invasiveness of the representative South African *S. aureus* isolates in 293 cells, expressed as a percentage relative to the invasive control, Cowan I.

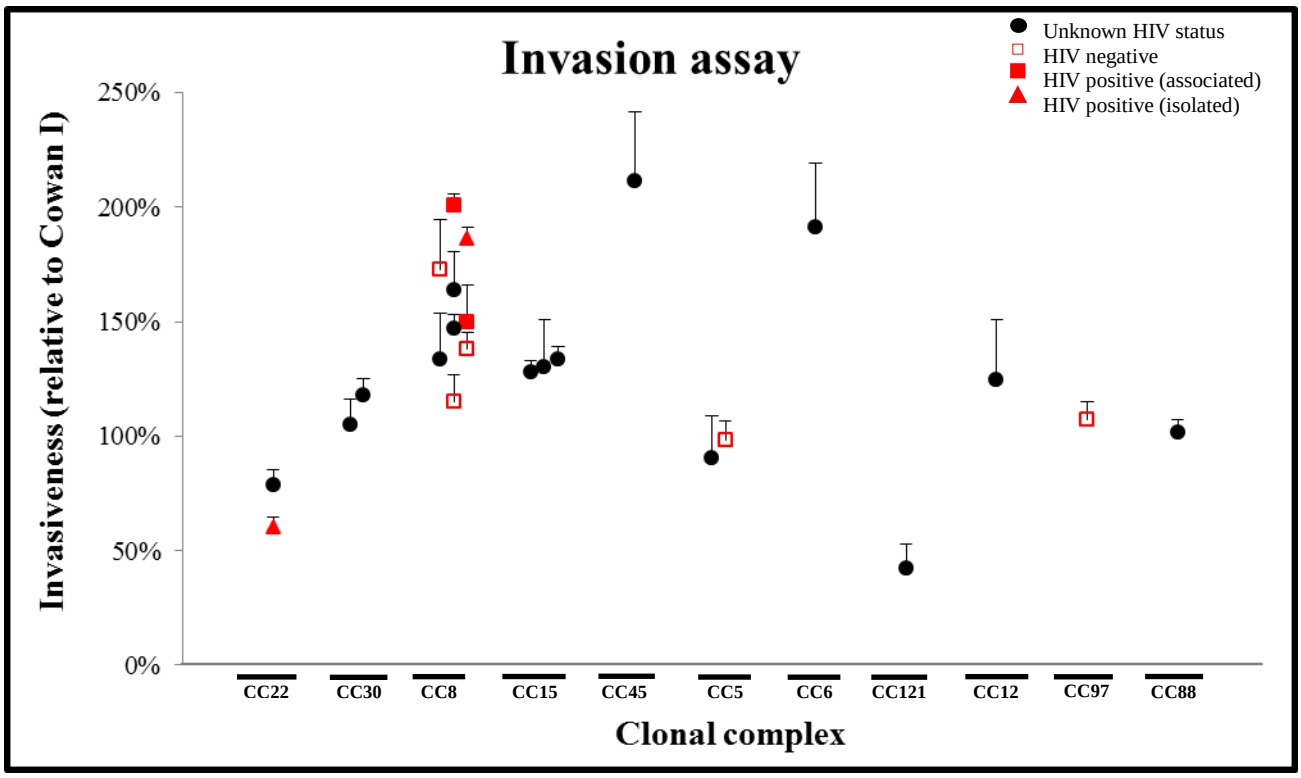


Figure 4.3 Invasiveness of representative South African *S. aureus* isolates grouped according to MLST CC and displayed according to HIV status.

4.4.2 Induction of host cell death:

The cell death induction abilities of the representative isolates were determined using three different assays. All three assays included cells-only controls and various chemical controls as inducers of cell death. Bacterial cytotoxic and non-cytotoxic controls were also included in every assay.

4.4.2.1 Nicoletti assay:

As can be seen in Figure 4.4, induction of host cell death of the 25 representative isolates was diverse (range 23.7% - 79.6% relative to the cells only control [set at 100% and inverted]; mean = 65.4%; median = 69.6%), although 21 of the isolates killed >60% of the cells they invaded.

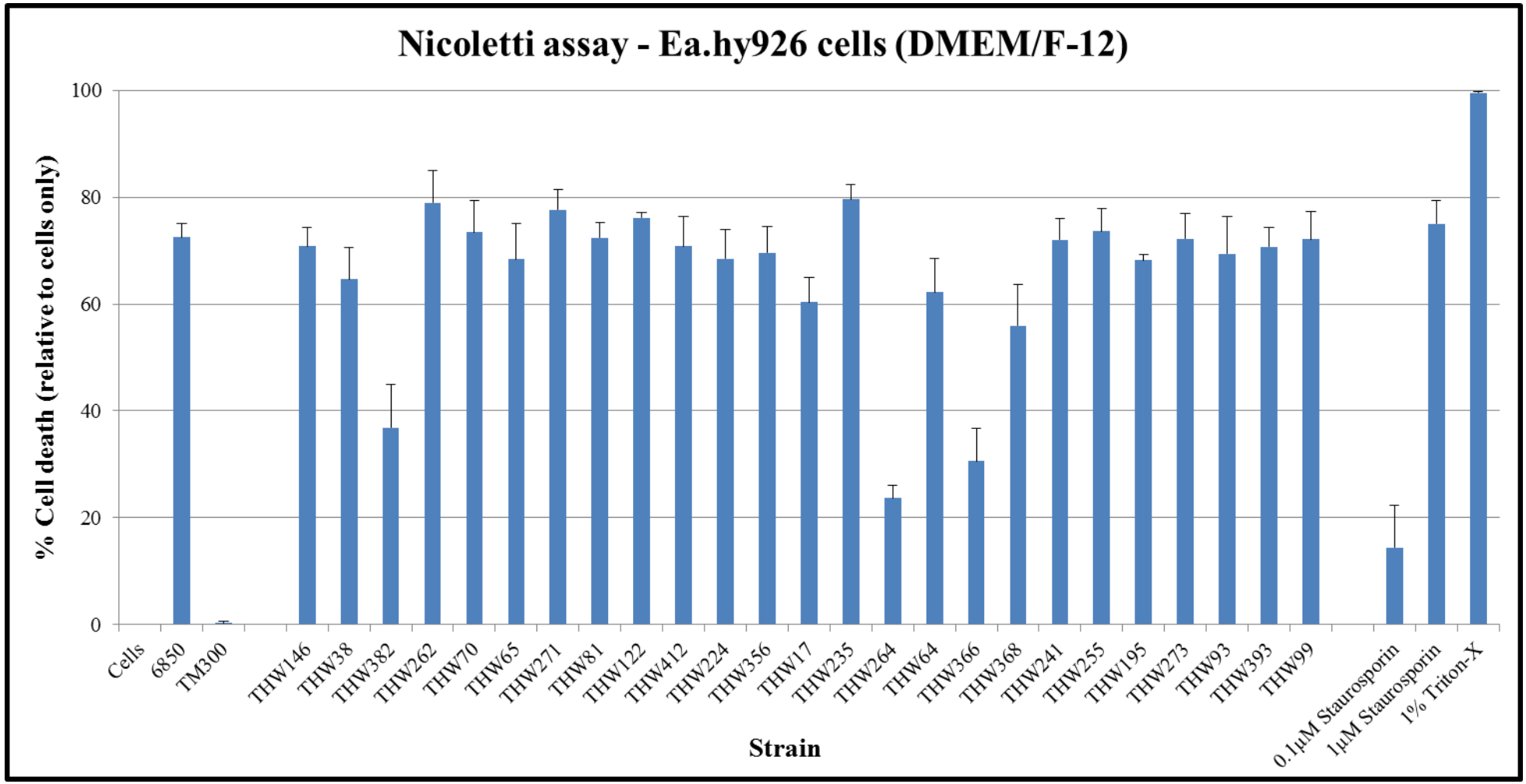


Figure 4.4 Differences in induction of host cell death of the representative South African *S. aureus* isolates in Ea.hy926 cells, expressed as a percentage relative to the cells only control.

Using an arbitrary cut-off value of 50% to distinguish non-cytotoxic from cytotoxic isolates, 22/25 isolates were cytotoxic.

The following three isolates were non-cytotoxic using this assay: (1) THW-366 (ST121:CC121; PVL+ MSSA; associated with SSTI); (2) THW-264 (ST5:CC5; SCC*mec* I; PVL- MRSA); and (3) THW-382 (ST36:CC30; SCC*mec* II; PVL- MRSA). Thirteen isolates could be classified as very cytotoxic (>70 % cell death) (CC22, CC8, CC15, CC5, CC97, CC88).

No significant difference in host cell death was observed between isolates from HIV+ or HIV- patients (Figure 4.5).

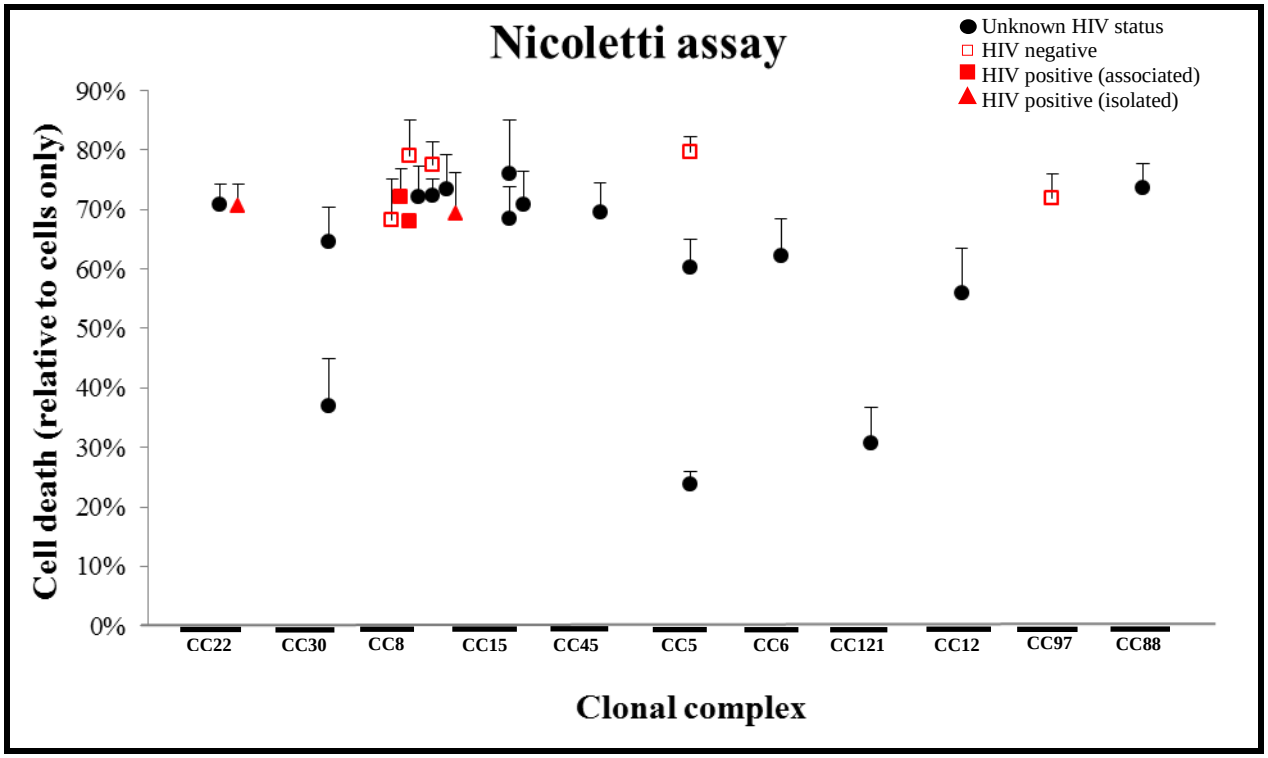


Figure 4.5 Induction of host cell death of representative South African *S. aureus* isolates grouped according to MLST CC and displayed according to HIV status.

The cytotoxicity of the non-cytotoxic control, TM300, was <10% relative to the cells only control, while that of the cytotoxic control, 6850, was >70%. The 1% Triton-X control displayed nearly 100% cytotoxicity, while a dose-dependent response was seen with the staurosporine controls, where the 1µM staurosporine control had a cytotoxic effect, while the 0.1µM staurosporine was non-cytotoxic.

4.4.2.2 LDH assay:

Induction of host cell death of the 25 representative isolates (as displayed in Figure 4.6), measured by the release of LDH into the culture supernatant, was also quite diverse (range 34.1% - 94.3% relative to the cytotoxic chemical control [1% Triton-X set at 100%]; mean = 61.4%; median = 59.6%).

Using an arbitrary cut-off value of 50% to distinguish non-cytotoxic from cytotoxic isolates, 22/25 isolates were cytotoxic and three non-cytotoxic, of which two were also classified as non-cytotoxic using the Nicoletti assay. The following three isolates were non-cytotoxic using this assay: (1) THW-366 (ST121:CC121; PVL+ MSSA; associated with SSTI); (2) THW-264 (ST5:CC5; SCC*mec* I; PVL- MRSA); and (3) THW-99 (ST239:CC8; SCC*mec* NT). Using this assay, 9 isolates could be classified as very cytotoxic (>70% cell death) (CC22, CC30, CC8, CC15, CC45, CC88).

No significant difference in host cell death was observed between isolates from HIV+ or HIV- patients (Figure 4.7).

The cytotoxicity of the non-cytotoxic control, TM300, was <10% relative to the 1% Triton-X control, while that of the cytotoxic control, 6850, was >75%. The 1% Triton-X control was set at 100% cytotoxic, while a dose-dependent response was seen with the staurosporine controls, where the 10µM staurosporine control had a cytotoxic effect, while the 1µM staurosporine was categorised as non-cytotoxic.

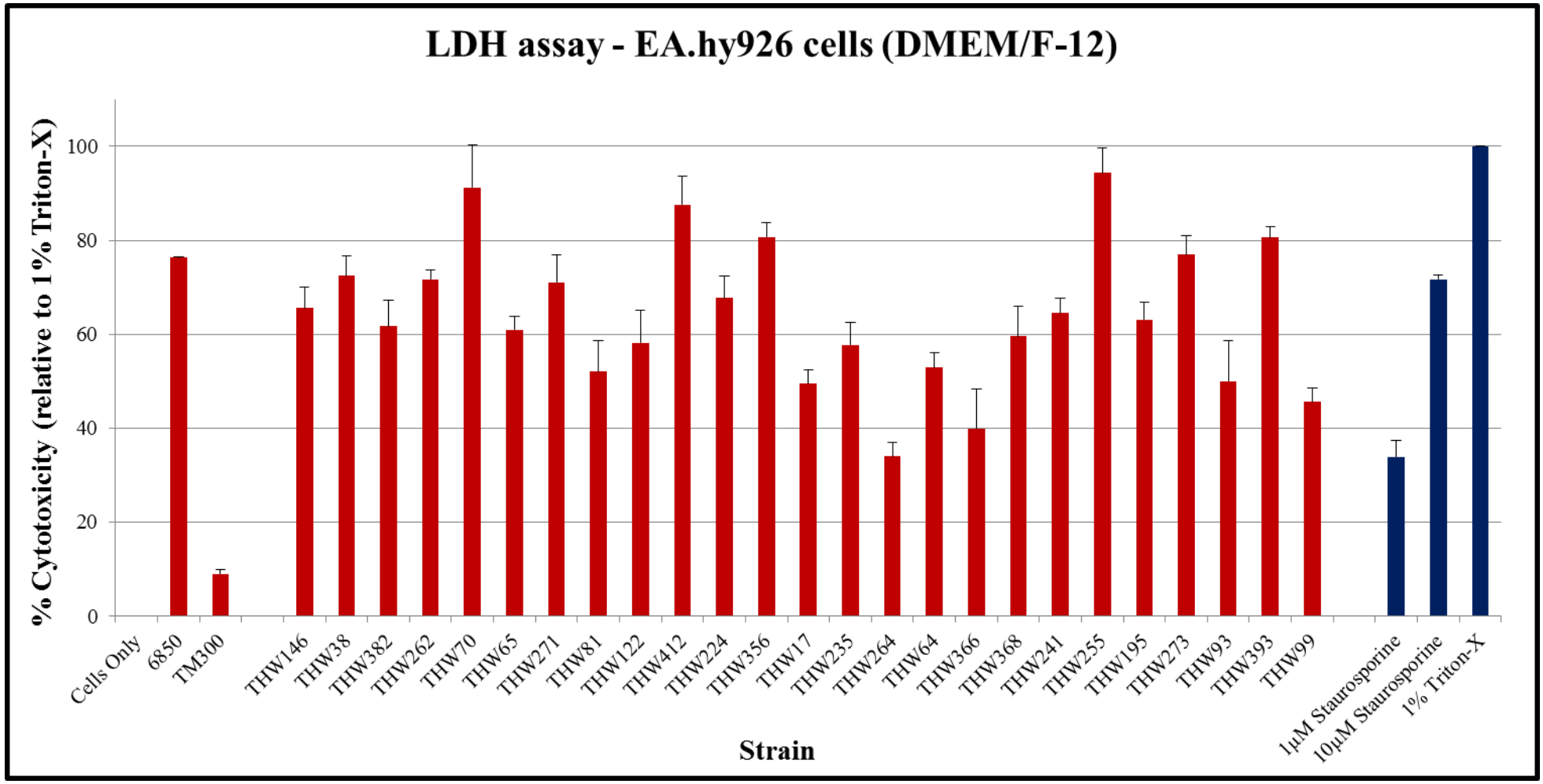


Figure 4.6 Differences in induction of host cell death of the representative South African *S. aureus* isolates in Ea.hy926 cells, expressed as a percentage relative to the cytotoxic chemical control, 1% Triton-X.

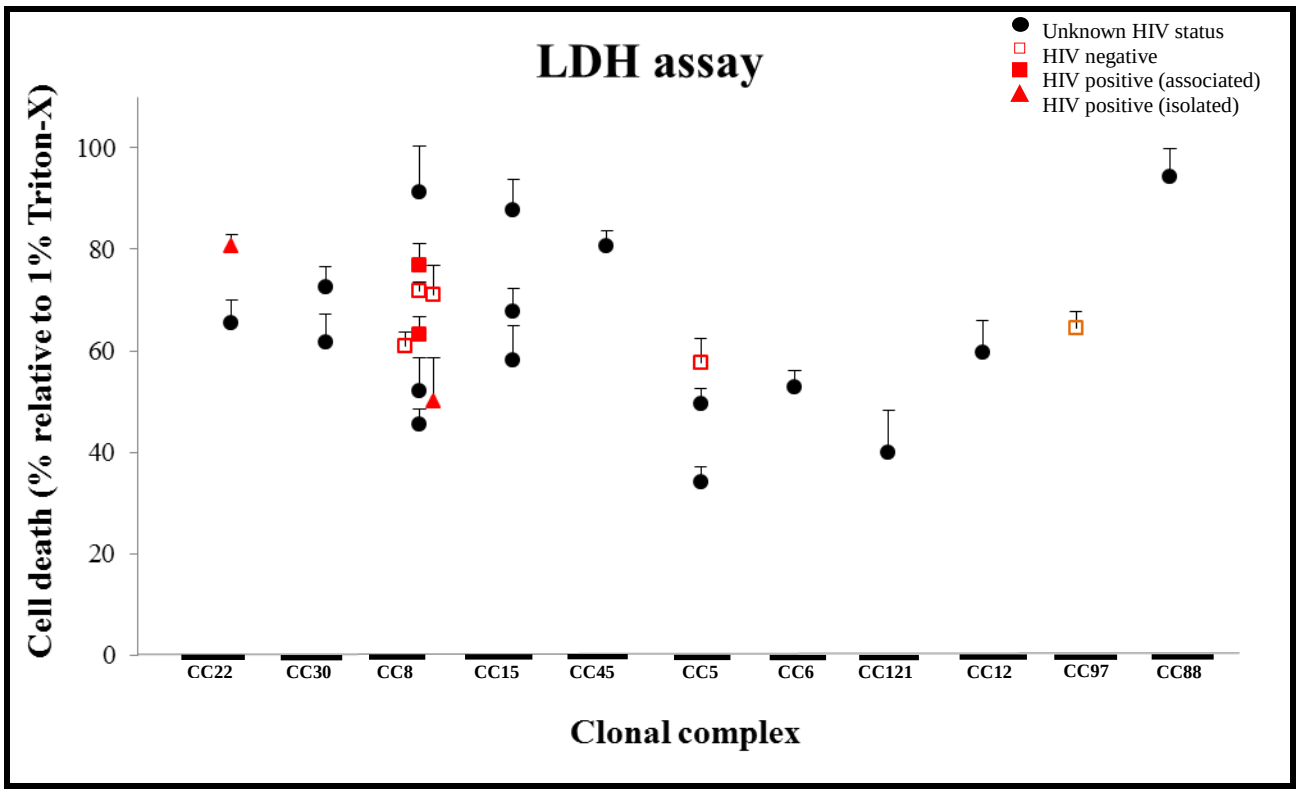


Figure 4.7 Induction of host cell death of representative South African *S. aureus* isolates grouped according to MLST CC and displayed according to HIV status.

4.4.2.3 WST-1 cell viability assay:

As can be seen in Figure 4.8, induction of host cell death of the 25 representative isolates, measured by the conversion of the chemical WST-1 by metabolically active mitochondria, was also quite diverse (range 68% - 27.1% relative to the cells only control [set at 100% and inverted]; mean = 38.1%; median = 36%).

Using an arbitrary cut-off value of 50% cell viability to distinguish non-cytotoxic from cytotoxic isolates, 22/25 isolates were cytotoxic and three were non-cytotoxic, of which the same two isolates were previously classified as non-cytotoxic using the Nicoletti and LDH assays. The following three isolates were non-cytotoxic using this assay: (1) THW-366 (ST121:CC121; PVL+ MSSA; associated with SSTI); (2) THW-264 (ST5:CC5; SCC*mec* I; PVL- MRSA); and (3) THW-81 (ST239:CC8; PVL- MRSA). Using this assay, 7 isolates could be characterised as very cytotoxic (<30% viable cells) (CC22, CC8, CC45, CC88).

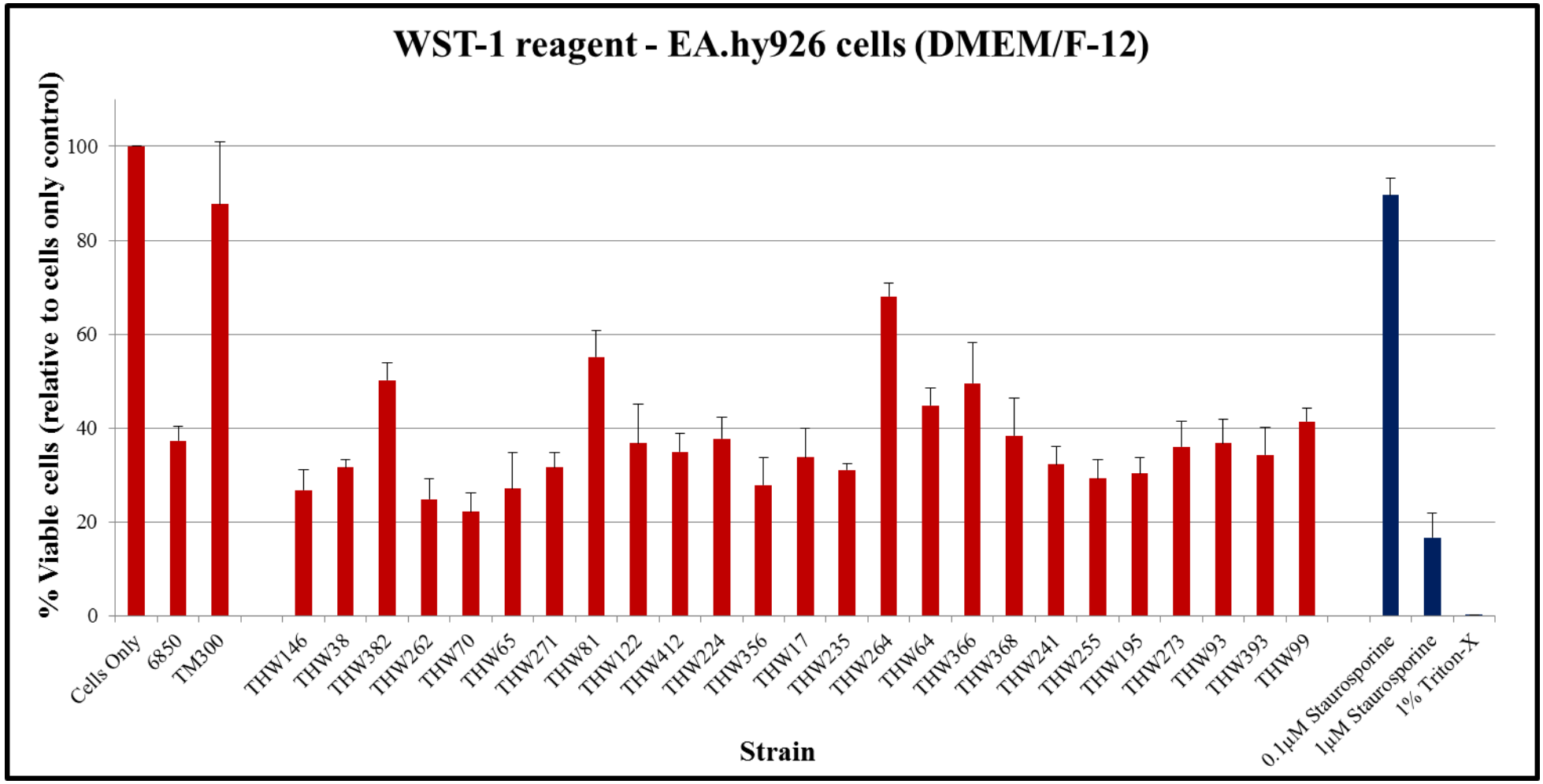


Figure 4.8 Differences in cell viability after induction of host cell death of the representative South African *S. aureus* isolates in Ea.hy926 cells, expressed as a percentage relative to the cells only control.

No significant difference in host cell death was observed between isolates from HIV+ or HIV- patients (Figure 4.9).

The cytotoxicity of the non-cytotoxic control, TM300, was >85% relative to the cells only control, while that of the cytotoxic control, 6850, was <60%. The 1% Triton-X control displayed 100% cytotoxicity, while a dose-dependent response was seen with the staurosporine controls, where the 1µM staurosporine control had a cytotoxic effect, while the 0.1µM staurosporine was non-cytotoxic.

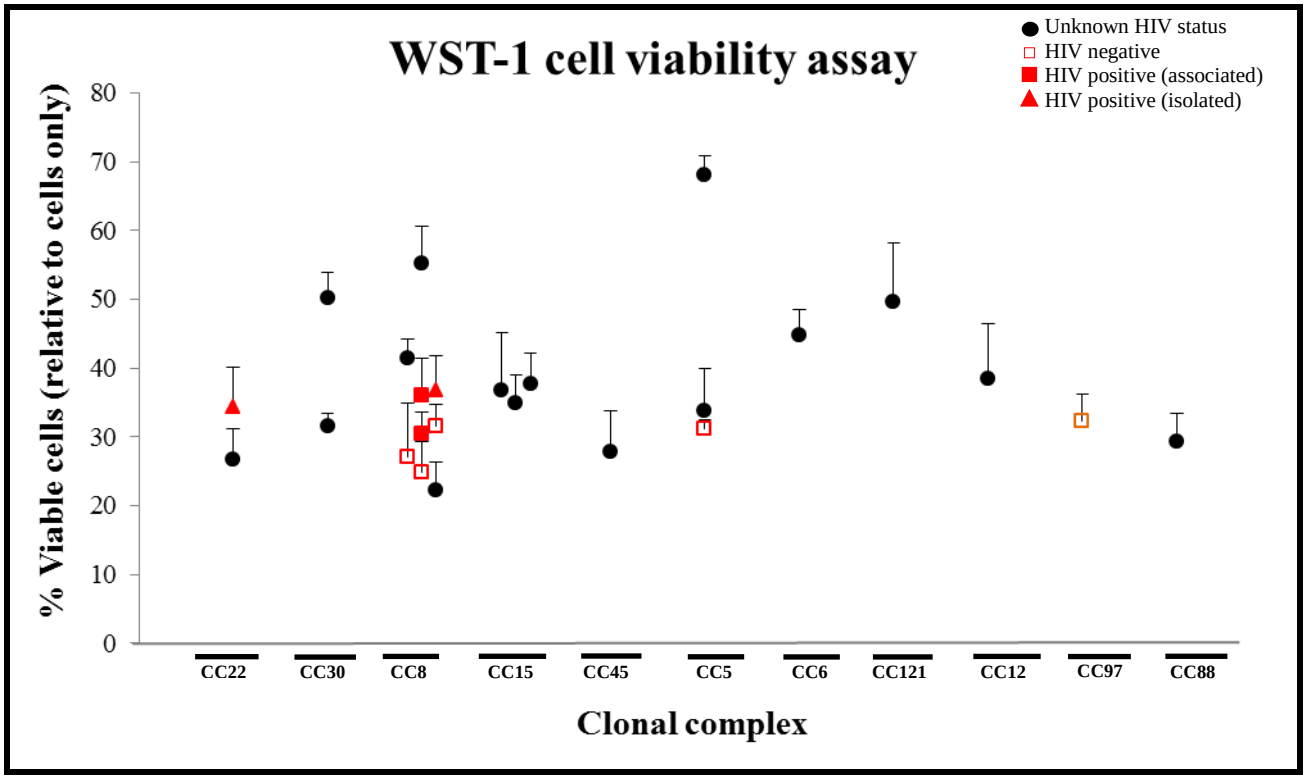


Figure 4.9 Cell viability after induction of host cell death of representative South African *S. aureus* isolates grouped according to MLST CC and displayed according to HIV status.

4.4.3 Statistical associations:

No association was identified between invasion or cell death and methicillin-resistance, using either the Mann-Whitney test or the 2-sample t-test, as displayed in Table 4.3, or between invasion or cell

death and patient HIV status (Table 4.5). No association was also identified between cell death and clonality (Table 4.4). CC8 isolates were the only isolates statistically associated with being invasive, displayed in Table 4.4.

Table 4.3 Statistical associations between invasion/cell death and methicillin-resistance.

Assay	Mann-Whitney test		2-sample t-test	
	Association with methicillin-resistance	**p-value**	**Association with methicillin-resistance**	**p-value**
Invasion	No association	0.681	No association	0.112
Nicoletti	No association	0.680	No association	0.901
LDH	No association	0.507	No association	0.910
WST-1	No association	0.577	No association	0.319

Table 4.4 Statistical associations between invasion/cell death and clonality. Statistically significant values are displayed in bold.

Assay	Kruskal-Wallis test	
	Association with clonality	**p-value**
Invasion	**CC8**	**0.036**
Nicoletti	No association	0.295
LDH	No association	0.324
WST-1	No association	0.706

Table 4.5 Statistical associations between invasion/cell death and patient HIV status.

Ligand	Kruskal-Wallis test	
	Association with HIV status	**p-value**
Invasion	No association	0.628
Nicoletti	No association	0.089
LDH	No association	0.829
WST-1	No association	0.101

PVL-negative isolates were more invasive than the PVL-positive isolates, which is displayed in Table 4.6.

Table 4.6 Statistical associations between invasion/cell death and bacterial PVL status. Statistically significant values are displayed in bold.

Ligand	Mann-Whitney test	
	Association with PVL status	**p-value**
Invasion	**Negative**	**0.004**
Nicoletti	No association	0.758
LDH	No association	0.586
WST-1	No association	0.525

4.5 Discussion

The research described in this chapter indicates that the majority of the isolates in this collection of representative strains were both invasive and cytotoxic to mammalian cells in the various *in vitro* assays we used.

4.5.1 Host cellular invasiveness:

We were able to establish that 23/25 isolates were invasive, irrespective of the clonal background of the isolate. Most isolates displayed invasiveness equal to the invasive control, Cowan I, while other isolates displayed invasiveness significantly more than that of the invasive control. Irrespective of the statistical test used, no association could be identified between invasion and methicillin-resistance. An association between clonality and invasiveness was only identified for CC8 isolates. But it should be taken into account that this was the only CC with enough isolates. CC8 isolates displayed the greatest diversity in cellular invasiveness, although all of them were classified as being invasive. The CC8 isolates also had the highest median value for invasion of all the CCs. The statistical associations are displayed in Tables 4.3 and 4.4 in section 4.4.3 and Figure 4.10 below.

Isolates of CC22, CC30, CC15 and CC5 displayed little variation in the degree of invasiveness. It is clear that for isolates from these CCs, being invasive is a clonal characteristic. Fowler *et al.* also previously reported that most *S. aureus* genotypes are capable of causing invasive diseases, but that CC5 and CC30 isolates displayed greater levels of haematogenous complication[313].

Being invasive is also a clonal characteristic for isolates from CC8. However, the isolates from this CC displayed a high degree of variation in invasiveness (range = 133.3% - 200.5%). We can therefore say that being invasive is a clonal trait, but the degree of invasiveness is a strain-specific characteristic.

We are unable to make similar conclusions from the data for isolates of CC45, CC6, CC12, CC97 and CC88 as only one isolate from each CC was characterised.

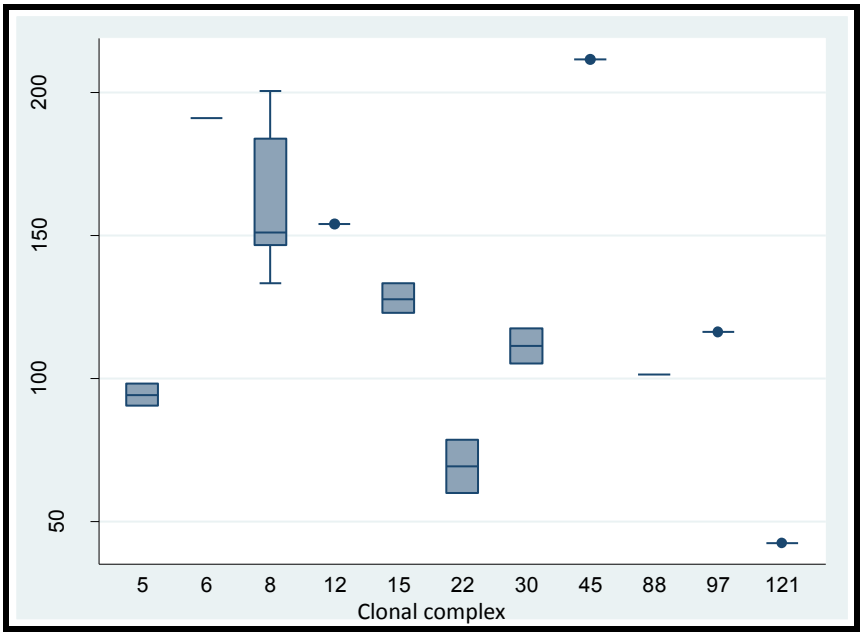


Figure 4.10 Kruskal-Wallis bar-graph of the cellular invasiveness of the representative isolates.

THW-366 (ST121; PVL+ MSSA; associated with SSTI; 42.4% invasiveness) was the only isolate classified as non-invasive and also the only *agr* IV isolate. This ST has previously been associated with CA-MRSA SSTI in children in Portugal[213], hospitalised patients in Russia[212] and rabbits from Belgium and Spain[214]. No data on the invasiveness was available from these studies. Our data also contradicts data published by Zautner *et al.*, who identified three ST121 isolates associated with recurrent tonsillitis. The isolates displayed invasiveness of 33.7%, 56.5% and 65.3% relative to Cowan I, the invasive control[286].

THW-264 (ST5:CC5; SCC*mec* I; PVL- MRSA) is another isolate of interest as we were unable to determine the invasiveness of this isolate due to technical difficulties. We can however speculate that this isolate might be *pls*$^{+}$, since this gene is commonly associated with SCC*mec* I. It has previously been demonstrated that MRSA isolates expressing *pls* displayed a significantly reduced ability to invade host cells[256, 257, 259]. We can hypothesise that this isolate might very likely be non-invasive. This isolate was collected from a paediatric patient suffering from conjunctivitis.

Zautner *et al.* identified a variety of CCs associated with recurrent tonsillitis-causing isolates as a result of intracellular persisting *S. aureus*. They characterised these isolates and identified many of them to be invasive isolates from CC30, CC45, CC8, CC5, CC15 and CC22[286]. Invasive isolates from the same clonal complexes were described during this study. However, this study was notr

designed to determine association of strains with a specific clinical syndrome, such as recurrent tonsillitis.

Park *et al.* investigated the association between invasiveness of clinical *S. aureus* isolates and their abilities to cause metastatic infections[314]. They found that invasiveness of endothelial cells is not a major determinant of metastatic infections.

When investigating the expression patterns of HUVEC cells upon internalisation of *S. aureus*, Stark *et al.* found that innate immune responses dominated, irrespective of the invasiveness of the bacterial isolates[315].

4.5.2 Induction of host cell death:

4.5.2.1 Nicoletti assay:

We were able to establish that 22/25 isolates were cytotoxic, irrespective of the clonal background of the isolate. Fourteen isolates displayed cytotoxicity equal to or more than the cytotoxic control, 6850, and were classified as very cytotoxic (>70% cell death). Using this assay, no isolate induced >80% cell death.

Isolates of CC22, CC8 and CC15 displayed little variation in the degree of cell death induction and it is clear that for these isolates, inducing the death of their host cells is a clonal characteristic.

Isolates from CC30 and CC5 displayed a high degree of diversity in their respective abilities to induce the death of their host cells (CC30 range = 37% - 64.6% cell death; CC5 range = 23.7% - 79.6%). For both CCs, some isolates were classified as being cytotoxic, while one isolate from every CC was classified as non-cytotoxic. Thus, the ability to induce the death of host cells for isolates of these CCs is a strain-specific characteristic.

We are unable to make similar conclusions from the data for isolates of CC45, CC6, CC12, CC97 and CC88 as only one isolate from every CC was characterised.

THW-366 (ST121; PVL+ MSSA; associated with SSTI; 30.6% cell death), the only isolate classified as non-invasive, was also classified as non/moderately cytotoxic using this assay. Based on the invasion data for the set of isolates, we can therefore say that invasion is required for the induction of cell death.

THW-264 (ST5:CC5; SCC*mec* I; PVL- MRSA), the isolate for which we could not determine invasiveness, was also classified as non/moderately cytotoxic using this assay (23.7% cell death). Actually, it was the least cytotoxic of all the representative isolates. If this isolate were to express *pls* as hypothesised earlier, it would fail to induce the death of host cells since its ability to invade the cells in the first place would be impeded.

The 3rd isolate classified as non/moderately cytotoxic was THW-382 (ST36:CC30; SCC*mec* II; PVL- MRSA), which was able to induce the death of only 36.9% of the cells using this assay, although this isolate was invasive (117.6%). The clone ST36-MRSA-II is usually associated with the clone EMRSA-16[23, 186], an epidemic MRSA clone. One would expect this clone and progeny thereof to be cytotoxic as this clone is globally spread. It is possible that this is the case as the Nicoletti assay measured hypodiploid nuclei and this isolate may induce host cell death employing pathways other than apoptosis, such as cell necrosis (61.7% cell death using LDH assay).

4.5.2.2 LDH assay:

We were able to establish that 22/25 isolates were cytotoxic, irrespective of the clonal background of the isolate. Nine isolates displayed cytotoxicity equal to or more than the cytotoxic control, 6850, and were classified as very cytotoxic (>70% cell death). Two isolates were regarded as extremely cytotoxic, as they induced the death of >90% of the cells (THW-70 [ST612:CC8; SCC*mec* IV; PVL- MRSA] and THW-255 [ST88:CC88; SCC*mec* IV; PVL+ MRSA]). Both isolates were isolated from SSTIs.

Although the isolates of CC22, CC30 and CC15 displayed variation in the degree of cell death induction, it is clear from this data that the ability to induce host cell death is a clonal trait, while the degree of cell death induction (cytotoxic vs. very cytotoxic vs. extremely cytotoxic) is a strain-specific characteristic.

Isolates from CC8 and CC5 displayed a high degree of diversity in their respective abilities to induce the death of their host cells (CC8 range = 45.6% - 91.2% cell death; CC5 range = 34.1% - 57.7%). For both CCs, the majority of isolates were classified as being cytotoxic, while one isolate from every CC was classified as non-cytotoxic. Thus, the ability to induce the death of host cells for isolates of these CCs is a strain-specific characteristic. CC8 isolates vary greatly in their abilities to induce host cell death.

We are unable to make similar conclusions from the data for isolates of CC45, CC6, CC12, CC97 and CC88 as only one isolate from every CC was characterised.

THW-366 (ST121; PVL+ MSSA; associated with SSTI; 40% cell death), the non-invasive isolate, was once again classified as non/moderately cytotoxic using this assay.

THW-264 (ST5:CC5; SCC*mec* I; PVL- MRSA), the isolate for which we could not determine invasiveness, was also classified as non/moderately cytotoxic using this assay (34.1% cell death). Again, it was the least cytotoxic of all the representative isolates using this assay. As previously hypothesised, the possible expression of *pls* would hinder this isolates' invasiveness, rendering it non-invasive and not able to induce host cell death.

The 3rd isolate classified as non/moderately cytotoxic was THW-99 (ST239:CC8; SCC*mec* NT), which was able to induce the death of only 45.6% of the host cells using this assay, although it was invasive (146.7%). It is possible that this isolate can induce host cell death using pathways other than cell necrosis, such as apoptosis (72.1% cell death using Nicoletti assay).

4.5.2.3 WST-1 cell viability assay:

We were able to establish that 22/25 isolates were cytotoxic, irrespective of the clonal background of the isolate. Seven isolates displayed cytotoxicity equal to or more than the cytotoxic control, 6850, and were classified as very cytotoxic (<30% cell viability).

Although the isolates of CC22 and CC30 displayed variation in the degree of cell viability, it is clear from this data that the ability to induce host cell death is a clonal trait. The same is true for the isolates from CC15, although they displayed very little variation.

Isolates from CC8 and CC5 displayed a high degree of diversity in their respective abilities to induce the death of their host cells (CC8 range = 55.2% - 22.2% cell viability; CC5 range = 68% - 31.1%). For both CCs, the majority of isolates were classified as being cytotoxic, while one isolate from every CC was classified as non-cytotoxic. Thus, the ability to induce the death of host cells for isolates of these CCs is a strain-specific characteristic, as isolates from both CCs vary greatly in their abilities to induce host cell death.

We are unable to make similar conclusions from the data for isolates of CC45, CC6, CC12, CC97 and CC88 as only one isolate from every CC was characterised.

THW-366 (ST121; PVL+ MSSA; associated with SSTI; 51% cell viability), the non-invasive isolate, was once again classified as non/moderately cytotoxic using this assay.

THW-264 (ST5:CC5; SCC*mec* I; PVL- MRSA), the isolate for which we could not determine invasiveness, was also classified as non/moderately cytotoxic using this assay (68% cell viability). Again, it was the least cytotoxic of all the representative isolates using this assay. As previously hypothesised, the possible expression of *pls* would hinder this isolates' invasiveness, rendering it non-invasive and not able to induce host cell death.

The 3rd isolate classified as non/moderately cytotoxic was THW-81 (ST239:CC8; SCC*mec* III; PVL- MRSA), which was able to induce the death of only 44.8% of the host cells using this assay, although it was invasive (146.7%). The reason why this isolate is classified as non-cytotoxic using this assay is unclear, since both of the previously discussed assays classified this isolate as cytotoxic (72.4% cell death using Nicoletti assay; 52% cell death using the LDH assay).

4.5.2.4 Combined host cell death induction data:

When combining all the data of the above discussed assays, it can be seen that 23/25 isolates are cytotoxic (>50% cell death using all three assays), irrespective of their genetic background, and the remaining two isolates are non-cytotoxic (<50% cell death using all three assays). Irrespective of the statistical test used, no association was identified between cell death and methicillin-resistance or between cell death and clonality. Please refer to Tables 4.3 and 4.4 in section 4.4.3.

Four isolates stood out as they were the only isolates classified as very cytotoxic according to all three assays, resulting in >70% cell death for every assay. This data shows that PVL+ isolates (THW146; THW393; THW255) can be as cytotoxic as PVL- isolates (THW262; THW70; THW271; THW273; THW412; THW356) in this setting, but PVL- very cytotoxic isolates are more common. A possible cause of this phenotypic display of high levels of cytotoxicity might be due to the action of PSMs.

The same applied for MSSA and MRSA isolates. Very cytotoxic MRSA and MSSA isolates were identified, and no association was identified between methicillin-resistance and cytotoxicity (refer to Table 4.3). Three very cytotoxic MRSA isolates were identified (THW262; THW70; THW255) and one very cytotoxic MSSA isolate was identified (THW356). Also, one non-cytotoxic MSSA and a MRSA isolate were identified.

It is interesting to note that the most cytotoxic MRSA isolates all carry SCC*mec* IV. For the ST88 isolate this makes sense as this ST is associated with CA-MRSA. ST612 has previously been described from hospital isolates in South Africa. This ST might have originated in the community and established itself later on in a health care setting. Table 4.7 provides more information on these isolates.

Table 4.7 Isolates classified as very cytotoxic according to all three assays.

Strain	Clone*	Cell death assay (% cell death)			Previous statistical associations identified
		Nicoletti	LDH	WST-1	
THW-262	ST612-MRSA-IV (THW-O)	79.0	71.7	75.1	MRSA/PVL-
THW-70	ST612-MRSA-IV (THW-N)	73.4	91.2	77.8	MRSA/PVL-
THW-356	ST45-MSSA (THW-EE)	70.0	80.7	72.2	MSSA/PVL-
THW-255	ST88-MRSA-IV (THW-BB)	73.6	94.3	70.7	MRSA/PVL+

* Clone name represented as [MLST-MSSA/MRSA-SCC*mec* (PFGE)]

Four isolates were classified as very cytotoxic according to the Nicoletti and LDH assays only (Table 4.8). We can speculate that these isolates are capable of inducing host cell death using both apoptotic and necrotic mechanisms. These isolates were identified from a pus swab of the tibia of an HIV+ man, fluid from a sinus fungal infection and pus from a hand infection from another HIV+ female.

Table 4.8 Isolates classified as very cytotoxic according to the Nicoletti and LDH assays only.

Strain	Clone*	Cell death assay (% cell death)		Previous statistical associations identified
		Nicoletti	LDH	
THW-393	ST22-MSSA (THW-C)	70.8	80.61	None
THW-271	ST8-MRSA-V (THW-J)	77.6	71.1	PVL-
THW-273	ST612-MRSA-IV (THW-P)	72.2	76.9	HIV+
THW-412	ST1-MSSA (THW-V2)	70.8	87.6	MSSA/PVL-

* Clone name represented as [MLST-MSSA/MRSA-SCC*mec* (PFGE)]

Five isolates were classified as very cytotoxic according to the Nicoletti assay only, of which PVL-MSSA isolates were most common (Table 4.9).

Table 4.9 Isolates classified as very cytotoxic according to the Nicoletti assay only.

Strain	Clone*	Cell death assay (% cell death)	Previous statistical associations identified
		Nicoletti	
THW-81	ST239-MRSA-III (THW-L)	72.4	MRSA/PVL-
THW-99^	ST239-MRSA-NT (THW-L)	72.1	None
THW-122	ST15-MSSA (THW-S)	76.1	MSSA/PVL-
THW-235	ST1864-MSSA (THW-Z)	79.6	MSSA/PVL+
THW-241	ST97-MSSA (THW-W)	72	MSSA/PVL-/Males

* Clone name represented as [MLST-MSSA/MRSA-SCC*mec* (PFGE)]; ^ = only NT SCC*mec*

One isolate was classified as very cytotoxic according to both the Nicoletti (70.8% cell death) and WST-1 (73.2% cell death) assays: THW-146 (ST22-MSSA [THW-C]), associated with SSTIs caused by PVL+ MSSA isolates, and also the dominant MSSA clone. One isolate was classified as very cytotoxic according to the LDH assay (72.4% cell death) only: THW-38 (ST1865-MSSA [THW-A]), associated with SSTIs caused by PVL+ MSSA isolates.

Park *et al.* investigated if any association was present between cytotoxicity of clinical *S. aureus* isolates and their abilities to cause metastatic infections[314]. They found that the ability to induce host cell death and be cytotoxic to endothelial cells was not a major determinant of metastatic infections.

During the investigation of 10 MRSA (CC45 and CC5) isolates collected during a national clinical trial, Seidl *et al.* determined that all 10 isolates were invasive in human endothelial cells, but only some of the isolates were cytotoxic[288]. They also identified an association between *in vitro* cell damage and bacterial virulence using an experimental rabbit infective endocarditis model, where infection by cytotoxic isolates resulted in higher bacterial densities in vegetations, kidneys and spleen.

Our data contradicts that of Krut *et al.*, who published cytotoxicity data of a collection of *S. aureus* isolates, which displayed cytotoxicity to be a strain-specific characteristic, and more than half of the collection of isolates were classified as non-cytotoxic[316].

4.6 Conclusion

The vast majority of clinical *S. aureus* isolates included in this study are invasive, although differences in the degree of invasiveness are common between different CCs. ST121 was the only ST in this collection classified as non-invasive.

The vast majority of these isolates are also able to induce the death of their host cells, irrespective of which time-point in the cell death cycle is measured.

No differences in either the cellular invasiveness or cytotoxicity of isolates from HIV+ and/or HIV- persons or PVL+ and/or PVL- isolates were detectable by this approach. Neither invasiveness nor cytotoxicity was statistically associated with methicillin-resistance, although the only isolate classified as non-invasive was a MRSA isolate carrying a SCC*mec* type I element.

CHAPTER 5: General discussion

During the previous three chapters (Chapter 2 to Chapter 4), we investigated the population structure of *S. aureus* from specific clinical sources and selected representative isolates for further analyses. We initiated analyses by determining the prevalence of selected virulence genes and their association with clonality and methicillin-resistance. This was followed by studying adherence of representative isolates to immobilised human ligands, as well as establishing their cellular invasiveness. The host-pathogen interaction assays were concluded by determining the cytotoxic potential of the investigated clones, as measured by three different end points for cell death. In this chapter, the statistical association of selected virulence factors of our representative isolates, as well as the statistical associations between the *in vitro* assays (adherence, invasion and cell death) for each individual clonal complex are reported and discussed. Finally, we investigated if any significant associations could be identified between the various *in vitro* assays used as surrogate parameters for virulence potential.

5.1 Population Structure:

Using a variety of molecular typing techniques, we were able to elucidate the epidemiology of *S. aureus* isolated from specific site infections at Tygerberg hospital. We were then able to go one step further, and investigated the population structure of the organism by associating dominant circulating clones with specific clinical and bacterial characteristics, such as clones associated with methicillin-resistance, and clones associated with adult or paediatric patients.

We identified 20 dominant clones, as determined by PFGE and supported by *spa*-CC and MLST, and were able to associate each clone with at least one clinical/bacterial characteristic, and one representative isolate was randomly selected from each of the 20 PFGE clones. Two PFGE clones were also associated with a HIV+ status and 1 representative isolate was selected from each one. A further two representative isolates were selected from isolates causing infection in HIV+ patients of

the dominant MRSA and MSSA clones, since these clones had the highest prevalence of HIV+ status among the patients, while the final representative isolate was selected as it was the only MRSA isolate which possessed a non-typeable SCC*mec* element.

Prevalence of selected virulence factors were first determined through PCR and then analysed for an association with specific clones and also methicillin-resistance. Certain virulence factors such as *fnb*A/B and *clf*A/B were identified in all the representative isolates. We were only able to investigate the association of virulence factors with clonality in CCs that included more than one isolate. Several virulence factors were found in CC22, CC30, CC5, CC8 and CC15. Table 5.1 provides a complete breakdown of the virulence factors associated with each isolate. The p-values obtained after investigation of the association of each individual virulence gene with clonality and methicillin-resistance are comprehensively described in Chapter 2, section 2.2.14 and an overview is presented in Table 5.1.

A very heterogenous population of clones was described, suggesting that only some MSSA lineages come into contact with SCC*mec* elements, or that only some MSSA lineages can successfully sustain the integration of SCC*mec* elements in the genome. The local acquisition of SCC*mec* elements by endemic MSSA clones was also reported during this study, including that of the dominant MRSA clone, ST612-MRSA-IV.

A prominent finding of this study is the high prevalence of PVL identified primarily among the MSSA clones. Many of these MSSA clones were also associated with SSTIs, similar to that of CA-MRSA strains described internationally. These PVL+ MSSA clones could potentially evolve and become CA-MRSA clones upon SCC*mec* acquisition. This study identified novel MRSA clones since strains from the same PFGE clonal complex with the same *spa* type, but opposing methicillin-resistance patterns, were identified. Thus, we hypothesis that these MRSA strains acquired their respective SCC*mec* elements locally. CA-MRSA clones are known to spread easily and can be associated with severe infection, such as necrotising fasciitis, which is a complicated infection and difficult to treat.

This study also identified a lack of CA-MRSA clones. Numerous SCC*mec* IV clones were described, but SCC*mec* IV is no longer regarded as a classic CA-MRSA element, since numerous SCC*mec* IV MRSA strains have adapted to hospital environments, such as the Paediatric clone[13].

Table 5.1 Representative isolates analysed, classified based on clonality, together with the clinical categories and virulence genes they are associated with.

Isolate name	PFGE: THW-	Source	MLST ST	MLST CC	*spa*-CC	SCC*mec*	*agr*	Statistical association	Virulence factors
THW38	A	SSTI	1865	30	21	NA	III	MSSA/PVL+/SSTI	*fnb*A/B; *clf*A/B; *eap; coa; nuc; hld; cap*8*; seg*; sem*; seo*; luk*S/F*
THW382	B	SSTI	36	30	21	II	III	MRSA/PVL-/Adults/SSTI	*fnb*A/B; *clf*A/B; *eap; coa; nuc; hld; cap*8*; see*; seg*; sem*; seo**
THW146	C	SSTI	22	22	891	NA	I	MSSA/PVL+/SSTI	*fnb*A/B; *clf*A/B; *eap; coa; nuc; hld; sdr*E*; cap*5*; seg*; sem*; seo*; luk*S/F*
THW393	C	BJ	22	22	891	NA	I	None[1]	*fnb*A/B; *clf*A/B; *eap; coa; nuc; hld; sdr*E*; cap*5*; seg*; sem*; seo*; luk*S/F
THW366	E	SSTI	121	121	NF:16	NA	IV	MSSA/PVL+/SSTI	*fnb*A/B; *clf*A/B; *eap; coa; nuc; hld; luk*S/F*
THW262	O	RT	612	8	64	IV	I	MRSA/PVL-	*fnb*A/B; *clf*A/B; *eap; coa; nuc; hld; sdr*C*; *sdr*E*; *cap*5*; *hlgv*; see*; sei**
THW65	G	BJ	1862	8	1597	NA	I	MSSA/Adults	*fnb*A/B; *clf*A/B; *eap; coa; nuc; hld; sdr*E*; *cap*5*; *hlgv**
THW271	J	ENT	8	8	64	V	I	PVL-/Adults	*fnb*A/B; *clf*A/B; *eap; coa; nuc; hld; cap*5*; *hlgv**
THW81	L	PROST	239	8	21	III	I	MRSA/PVL-	*fnb*A/B; *clf*A/B; *eap; coa; nuc; hld; sdr*C*; *hlgv*; sei**
THW70	N	SSTI	612	8	64	IV	I	MRSA/PVL-	*fnb*A/B; *clf*A/B; *eap; coa; nuc; hld; sdr*C*; *sdr*E*; *cap*5*; *hlgv*; see*; sei**
THW195	K	SSTI	8	8	64	NA	I	HIV+	*fnb*A/B; *clf*A/B; *eap; coa; nuc; hld; sdr*C*; *sdr*E*; *cap*5*; *hlgv*; sei**
THW273	P	SSTI	612	8	64	IV	I	HIV+	*fnb*A/B; *clf*A/B; *eap; coa; nuc; hld; sdr*C*; *sdr*E*; *cap*5*; *hlgv*; see*; sei**
THW93	O	UK	612	8	64	IV	I	None[1]	*fnb*A/B; *clf*A/B; *eap; coa; nuc; hld; sdr*C*; *sdr*E*; *cap*5*; *hlgv*; see*; sei**
THW99	L	SSTI	239	8	21	UK	III	None[2]	*fnb*A/B; *clf*A/B; *eap; coa; nuc; hld; sdr*C*; *hlgv*; see*; sei**
THW122	S	SSTI	15	15	84	NA	II	MSSA/PVL-	*fnb*A/B; *clf*A/B; *eap; coa; nuc; hld; sdr*E*; *cap*5*; *hlgv**
THW412	V2	UT	1	15	NF:14	NA	III	MSSA	*fnb*A/B; *clf*A/B; *eap; coa; nuc; hld; sdr*E*; *cap*5*; *hlgv**
THW224	X	SSTI	188	15	EX	NA	I	MSSA/PVL-	*fnb*A/B; *clf*A/B; *eap; coa; nuc; hld; sdr*E*; *cap*5*; *hlgv**
THW17	Y	SSTI	1863	5	2	NA	II	MSSA/PVL-	*fnb*A/B; *clf*A/B; *eap; coa; nuc; hld; sdr*E*; *cap*5*; *hlgv*; seg*; sem*; seo**
THW235	X	EY	1864	5	2	NA	II	MSSA	*fnb*A/B; *clf*A/B; *eap; coa; nuc; hld; sdr*E*; *cap*5*; *hlgv*; seg*; sem*; seo*; luk*S/F*
THW264	AA	EY	5	5	2	I	II	MRSA/PVL-	*fnb*A/B; *clf*A/B; *eap; coa; nuc; hld; sdr*C*; *cap*5*; *hlgv*; seg*; sei*; sem*; seo**
THW64	V1	CA	6	6	64	NA	I	MSSA/PVL-	*fnb*A/B; *clf*A/B; *eap; coa; nuc; hld*
THW356	EE	SSTI	45	45	EX	NA	I	MSSA/PVL-	*fnb*A/B; *clf*A/B; *eap; coa; nuc; hld*
THW368	V3	SSTI	12	12	888	NA	II	MSSA/PVL+	*fnb*A/B; *clf*A/B; *eap; coa; nuc; hld; luk*S/F*
THW241	W	IVD	97	97	267	NA	I	MSSA/PVL-/Males	*fnb*A/B; *clf*A/B; *eap; coa; nuc; hld*
THW255	BB	SSTI	88	88	186	IV	III	PVL+/SSTI	*fnb*A/B; *clf*A/B; *eap; coa; nuc; hld; luk*S/F*

* = statistical association (see Table 2.16, Chapter 2); SSTI = skin and soft tissue infection; BJ = bone-and-joint; RT = respiratory tract; ENT = ear, nose and throat; PROST = prosthetic device; UK = unknown; UT = Urinary tract; EY = eye; CA = cardiac; IVD = intra-vascular device; 1 = collected from HIV+ patient of dominant MSSA and MRSA clone; 2 = only MRSA with non-typeable SCC*mec*, *fnb*A/B = fibronecting binding protein A/B gene,

*clf*A/B = clumping factor A/B gene; *eap* = extracellular edherence protein gene; *coa* = coagulase gene; *nuc* = nuclease gene; *hld* = delta toxin gene; *sdr*C/D/E = Sdr repeat protein gene C/D/E, *luk*S/F = Panton-Valentine leucofidin genes S and F; *hlg*v = gamma toxin variant gene; *cap*5/8 = capsule gene 5/8; *see/g/m/o/i* = staphylococcal enterotoxin E/G/M/O/I genes.

5.2 *In Vitro* Host Cell Interactions:

Following the identification of genes coding for selected virulence factors, we investigated how the representative isolates interacted with host proteins and host cells *in vitro*. We examined their ability to adhere to specific human ligands, their cellular invasiveness and their abilities to induce the death of cells, using cell-culture experiments. These results have been combined and will be discussed in more detail below for each isolate selected as a representative of its clonal background.

5.2.1 *In vitro* interactions per clonal complex:

As previously discussed in Chapter 3, strain-specific adherence pattern was noticed for the CC22 isolates, where THW146 displayed stronger adherence to plasma fibronectin, fibrinogen and both collagens tested as compared to THW393, the other CC22 isolate (Figure 5.1). Irrespective of the differences noticed in the isolates' adherence patterns, both could be classified as being invasive and cytotoxic in all the cell death assays used. The *sdr*E gene, hypothesised to be involved in adherence, was also associated with the isolates of this CC.

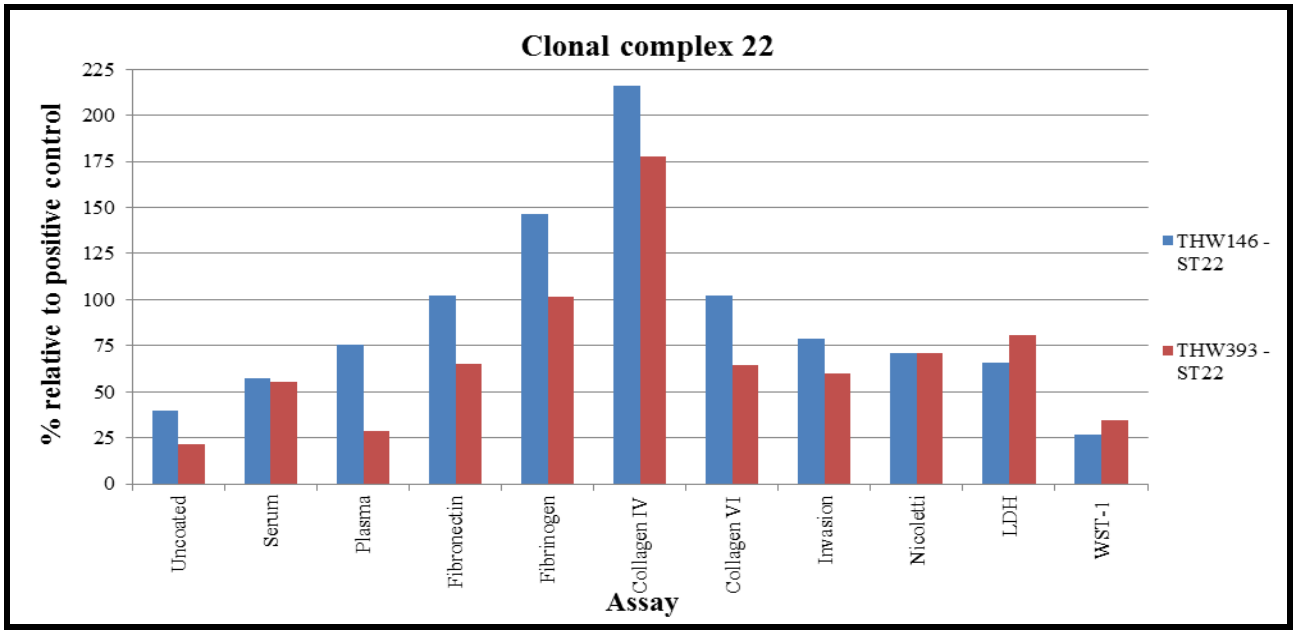


Figure 5.1 Combined results of all the *in vitro* assays for CC22 isolates.

The *fnb*A/B and *eap* genes, required for invasion, were identified in both isolates, as well as *hla* and *hld*, associated with cell death.

Regarding Figures 5.1 – 5.6, "uncoated, serum plasma, fibronectin, fibrinogen, collagen IV and collagen VI" refers to adherence, "invasion" refers to cellular invasion and "Nicoletti, LDH and WST-1" refers to cell death.

Statistical association between clonality and adherence, invasion or cell death is displayed in Table 5.2 and has previously been discussed in Chapter 3 and Chapter 4.

Table 5.2 Statistical associations between adherence/invasion/cell death and clonality. Statistically significant results ($p < 0.05$) are displayed in bold.

Parameter	**Assay**	**Kruskal-Wallis test**	
		Association with clonality	**p-value**
Adherence	Uncoated	No association	0.194
	Serum	No association	0.152
	Plasma	No association	0.530
	Fibronectin	No association	0.201
	Fibrinogen	No association	0.160
	Collagen IV	No association	0.248
	Collagen VI	No association	0.292
Invasion	**Invasion**	**CC8**	**0.036**
Cell death	Nicoletti	No association	0.295
	LDH	No association	0.324
	WST-1	No association	0.706

Regarding the CC30 isolates (Figure 5.2), significant differences were observed in the ability of isolates to adhere to collagen IV and VI, where THW38 (ST1864-MSSA) adhered much stronger to these two ligands than THW382 (ST36-MRSA-II). Both isolates were also classified as being invasive. Some diversity was identified relating to the cell death assays. Both isolates were classified as being cytotoxic using the LDH and WST-1 assays, but only THW38 was classified as cytotoxic using the Nicoletti assay. The *fnb*A/B and *eap* genes, required for invasion, were identified in both isolates, as well as *hla* and *hld*, associated with cell death.

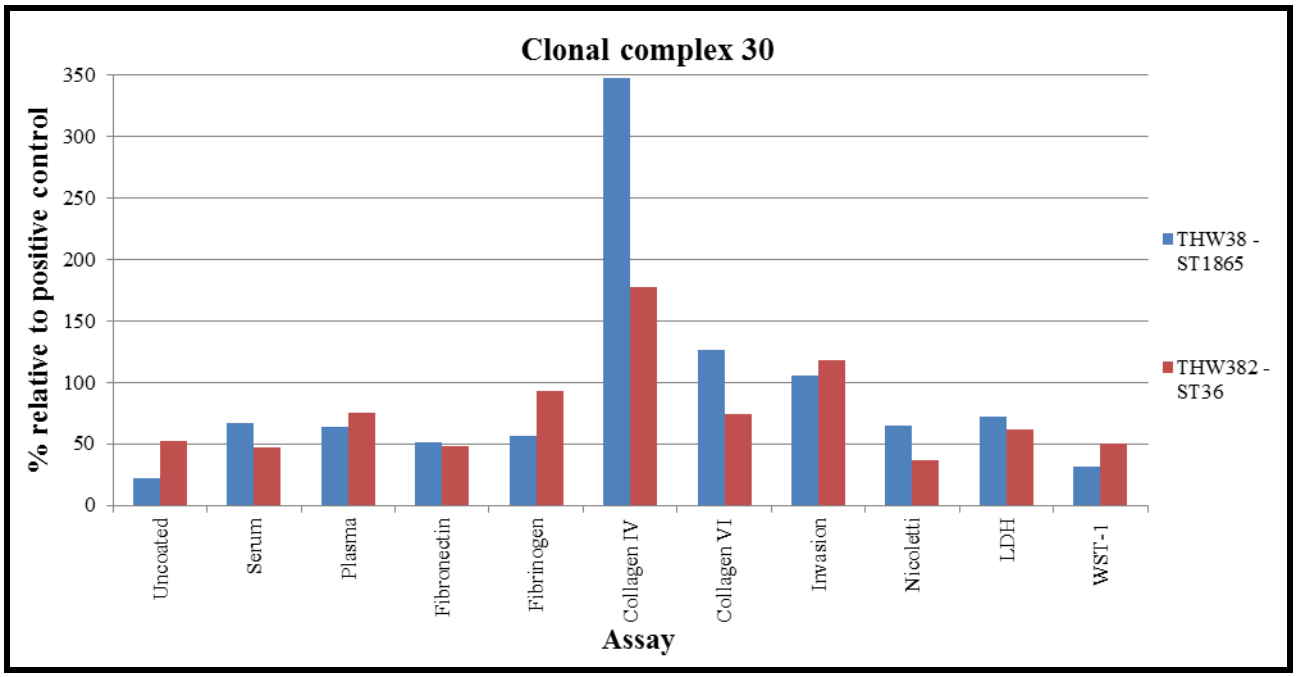


Figure 5.2 Combined results of all the *in vitro* assays for CC30 isolates.

Some diversity was also noticed regarding adherence of CC5 isolates (Figure 5.3). THW17 (ST1863-MSSA) displayed the strongest adherence towards serum, fibronectin, collagen IV and collagen VI, while THW235 (ST1865-MSSA) displayed the strongest adherence towards fibrinogen. THW264 (ST5-MRSA-I) displayed adherence towards plasma only, and was also the only isolate for which we could not establish cellular invasiveness. THW264 was also one of 2 isolates that was classified as non-cytotoxic. The remaining two CC5 isolates, THW17 and THW235, displayed invasiveness similar to that of the control isolate (Cowan I) and both were classified as cytotoxic. The *sdr*E gene was associated with the MSSA isolates (THW17 and THW235) from this CC, while *sdr*C was associated with the MRSA isolate, THW264. This was also the only isolate in which *hla* was not identified and was classified as non-cytotoxic. *hlg*v was

also associated with all isolates from this CC, while *fnb*A/B and *eap*, required for invasion, were identified in all isolates, as well as *hld*, associated with cell death.

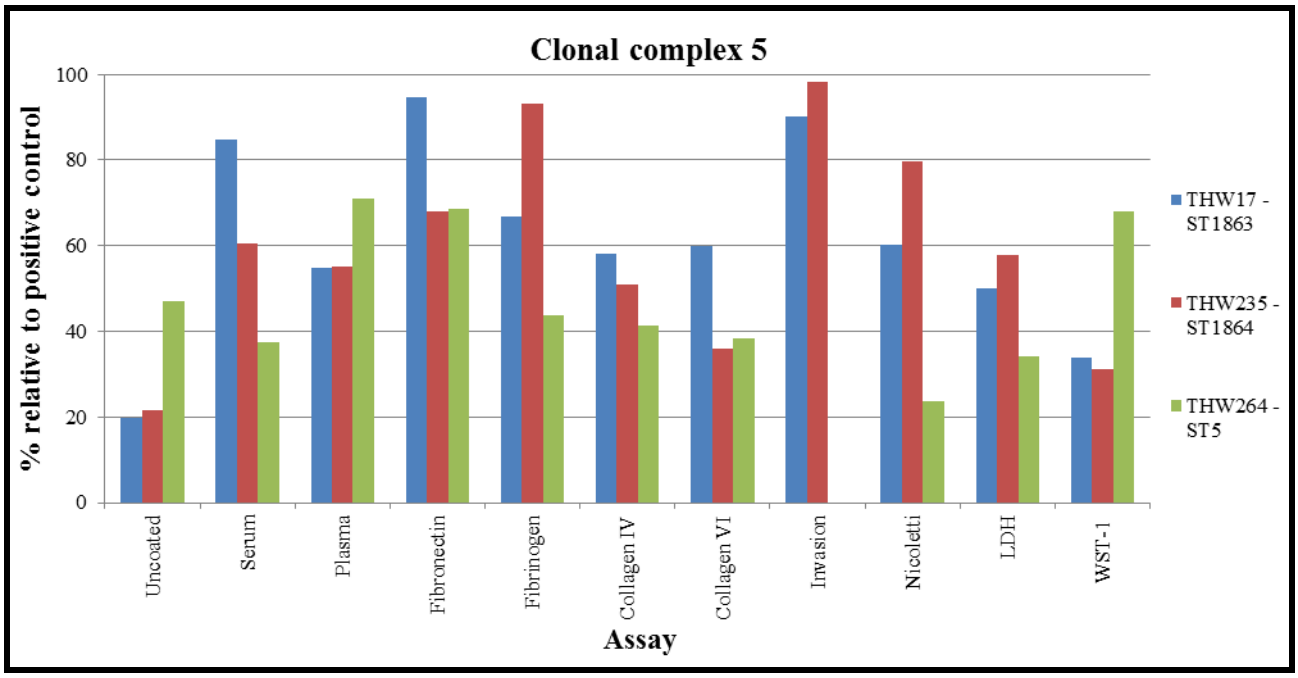


Figure 5.3 Combined results of all the *in vitro* assays for CC5 isolates.

Two isolates from CC15 displayed extremely strong adherence towards collagen IV and strong adherence towards collagen VI (Figure 5.4). THW412 (ST1-MSSA) adhered to collagen IV five times stronger than the control isolate (NCTC8325-4), while THW224 (ST188-MSSA) displayed adherence 3 times stronger. All 3 isolates were classified as invasive and cytotoxic. The *sdr*E gene was associated with all isolates of this CC, together with *hlgv*. *fnb*A/B and *eap*, required for invasion, were identified in all isolates, as well as *hla* and *hld*, associated with cell death.

As previously discussed, THW64 (ST6-MSSA), THW366 (ST121-MSSA) and THW368 (ST12-MSSA) displayed extreme preference for adherence to collagen IV and strong adherence to collagen VI (Figure 5.5). All singleton STs were classified as invasive and cytotoxic, except for the ST121 isolate, THW366. The *fnb*A/B and *eap* genes, required for invasion, were identified in all isolates, as well as *hla* and *hld*, associated with cell death. Even though *hla* was identified in THW366, this isolate was classified as non-cytotoxic.

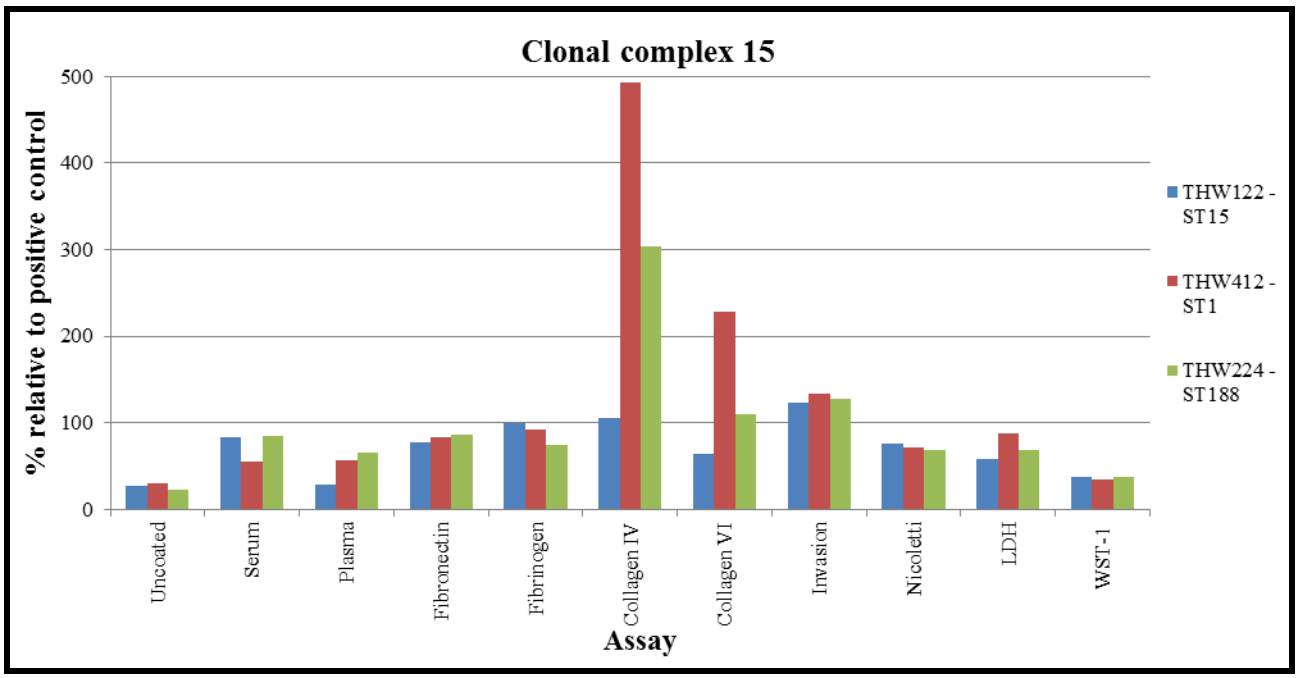


Figure 5.4 Combined results of all the *in vitro* assays for CC15 isolates.

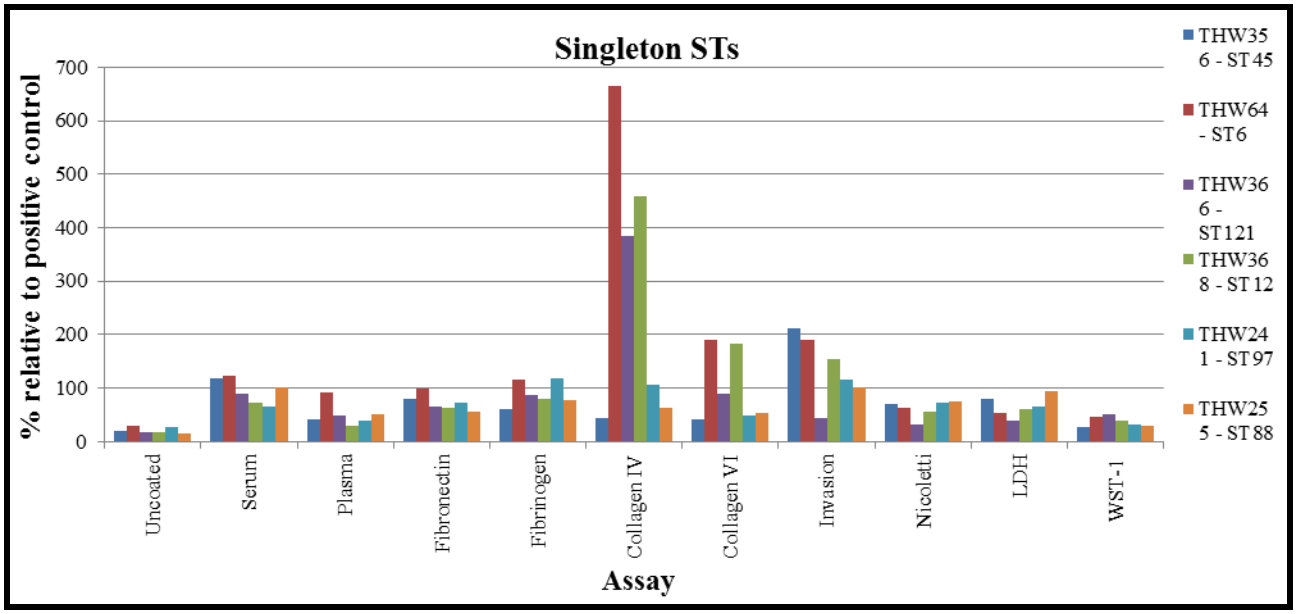


Figure 5.5 Combined results of all the *in vitro* assays for the singleton STs.

Diversity was noticed regarding adherence to the various ligands for the isolates from CC8 (Figure 5.6). As previously discussed, the ST612-MRSA-IV isolates (THW262, THW70, THW273 and THW93) displayed strong adherence towards plasma, fibronectin and collagen IV as well as

extremely strong adherence towards fibrinogen. The *sdr*C and *sdr*E genes was associated with all the ST612-MRSA-IV isolates.

The ST239 isolates, THW81 (ST239-MRSA-III) and THW99 (ST239-MRSA-NT) displayed extreme preference towards collagen IV and collagen VI (Figure 5.6). All the CC8 isolates were classified as being highly invasive and cytotoxic. It is worth noting that the 2 ST239 isolates were classified as being cytotoxic by only 2 of the 3 cell death assays used. The *sdr*C gene was associated with both ST239 isolates.

The *sdr*C and *sdr*E genes was also only associated with 1/2 ST8 isolates, THW195, while *sdr*E was associated with the only ST1862 isolate. The *fnb*A/B and *eap* genes, required for invasion, were identified in all isolates, as well as *hla* and *hld*, associated with cell death.

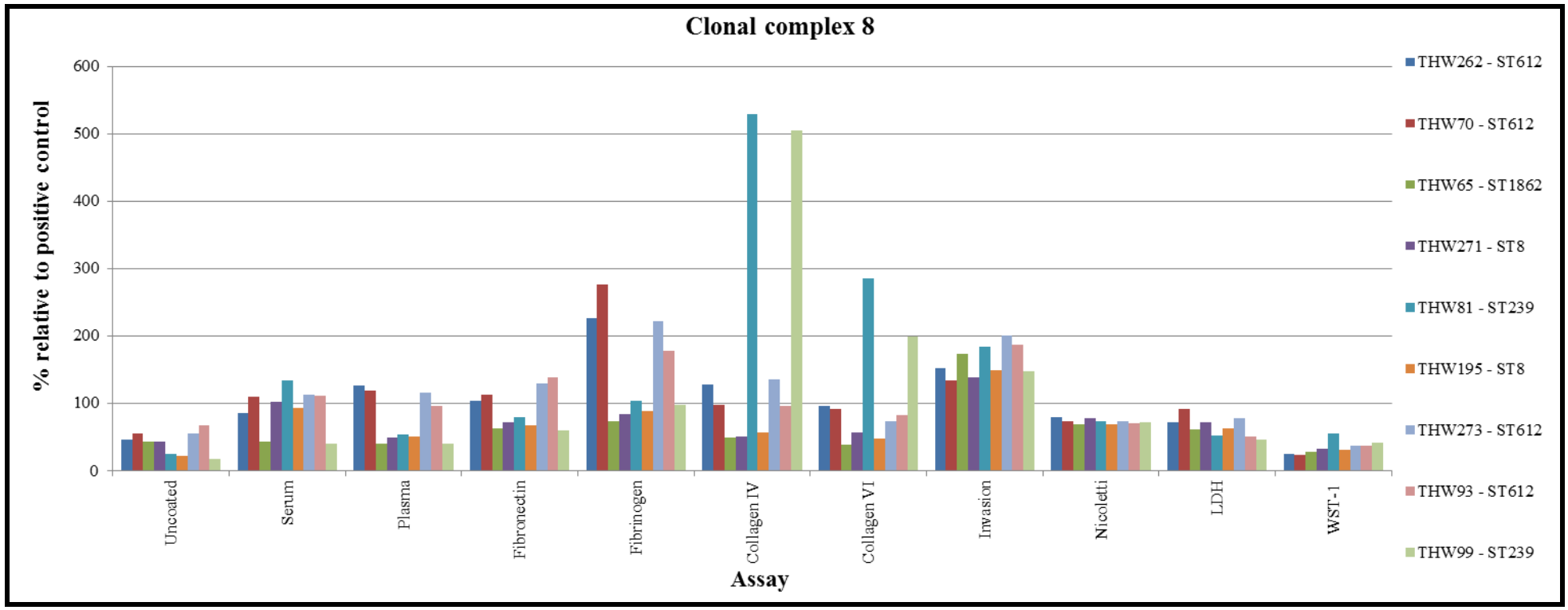


Figure 5.6 Combined results of all the *in vitro* assays for CC8 isolates.

5.2.2 *In vitro* correlation analyses:

Table 5.3 Pearson correlation (r) for the correlation between different assays and the corresponding p-value, calculated using the software package STATISTICA v.10. Statistically significant ($p < 0.05$) correlations are displayed in bold.

Assay	Pearson correlation (r)	p-value	Figure
Correlation between different adherence assays			
Uncoated vs. serum	0.0498	0.830	5.7
Uncoated vs. plasma	**0.7046**	**0.00008**	**5.8**
Uncoated vs. fibronectin	**0.6025**	**0.0014**	**5.9**
Uncoated vs. fibrinogen	**0.6277**	**0.0008**	**5.10**
Uncoated vs. collagen IV	-0.3363	0.102	5.11
Uncoated vs. collagen VI	-0.2131	0.3065	5.12
Plasma vs. serum	0.3256	0.1123	5.13
Plasma vs. fibronectin	**0.7099**	**0.00007**	**5.14**
Plasma vs. fibrinogen	**0.7842**	**0.000001**	**5.15**
Plasma vs. collagen IV	-0.0596	0.7771	5.16
Plasma vs. collagen VI	0.0122	0.9538	5.17
Serum vs. fibronectin	**0.4997**	**0.0110**	**5.18**
Serum vs. fibrinogen	0.3481	0.0882	5.19
Serum vs. collagen IV	0.0791	0.7069	5.20
Serum vs. collagen VI	0.1674	0.4238	5.21
Fibronectin vs. fibrinogen	**0.7439**	**0.00002**	**5.22**
Fibronectin vs. collagen IV	-0.0852	0.6854	5.23
Fibronectin vs. collagen VI	0.0339	0.8721	5.24
Fibrinogen vs. collagen IV	-0.1055	0.6157	5.25
Fibrinogen vs. collagen VI	0.0195	0.9262	5.26
Collagen IV vs. collagen VI	**0.9048**	**0.00001**	**5.27**
Correlation between adherence and invasion assays			
Uncoated vs. invasion	0.3279	0.1179	5.28
Serum vs. invasion	**0.5397**	**0.0065**	**5.29**
Plasma vs. invasion	0.3923	0.0579	5.30
Fibronectin vs. invasion	**0.4702**	**0.0204**	**5.31**
Fibrinogen vs. invasion	0.3121	0.1376	5.32
Collagen IV vs. invasion	0.1054	0.6241	5.33
Collagen VI vs. invasion	0.2418	0.2549	5.34

5.2.2.1 Correlations between adherence assays (ligands):

Strong correlations (r value ≥ 0.7) were identified between adherence in the absence of a ligand (uncoated plates) and plasma (Pearson correlation r = 0.7046, p = 0.00008), fibronectin (r = 0.6025, p = 0.0014) and fibrinogen (r = 0.6277, p = 0.0008), which were all significant. This might suggest that the charge of the uncoated plate promotes adherence or that the same surface components are involved in adherence in the absence as well as presence of these ligands. Strong correlations were also identified between plasma and fibronectin (r = 0.7099, p = 0.00007) and fibrinogen (r = 0.7842, p = 0.0000015), which is expected as human plasma is a rich source of both proteins. The receptors involved in adherence to fibronectin (*fnb*A/B) and fibrinogen (*clf*A/B) are probably also involved in adherence to human plasma protein coated surfaces. Adherence to fibronectin (r = 0.4997, p = 0.0110) also strongly correlated with that of serum, which is also expected as serum is rich in fibronectin. Adherence to fibronectin (r = 0.7439, p = 0.00002) correlated well with that of fibrinogen. Adherence to collagen IV or VI showed no significant correlation with any of the other ligands, except each other (r = 0.9048, p = 0.00001). This might argue for a common adhesin for both collagens, or strict co-regulation of the genes. Data suggests no association between *cna* gene prevalence and adherence to collagen (see Chapter 3, Appendix B, Figures B6 and B7). r and p-values are listed in Table 5.3 and the scatter plots of the results correlated are illustrated by Figures 5.7 – 5.27.

Statistical trends can be identified between adherence in the absence of a ligand and collagen IV (r = -0.336. p = 0.1) where an indirect correlation was identified. A trend also emerged regarding adherence to serum and fibrinogen (r = 0.348, p= 0.09). This can be expected since human serum is rich in fibrinogen.

Regarding all scatter plots throughout this chapter: the solid red line is the correlation curve and the dotted red lines indicate the 95% CI area.

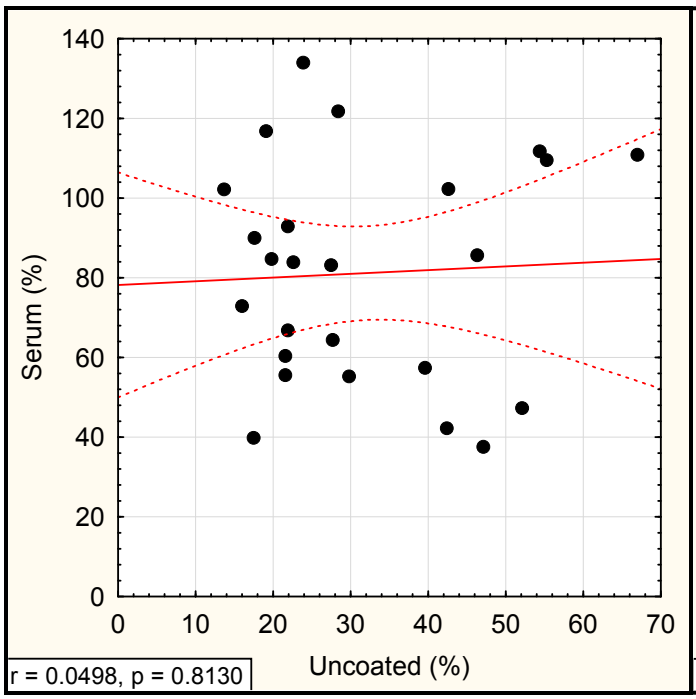


Figure 5.7 Scatter plot of uncoated vs. serum.

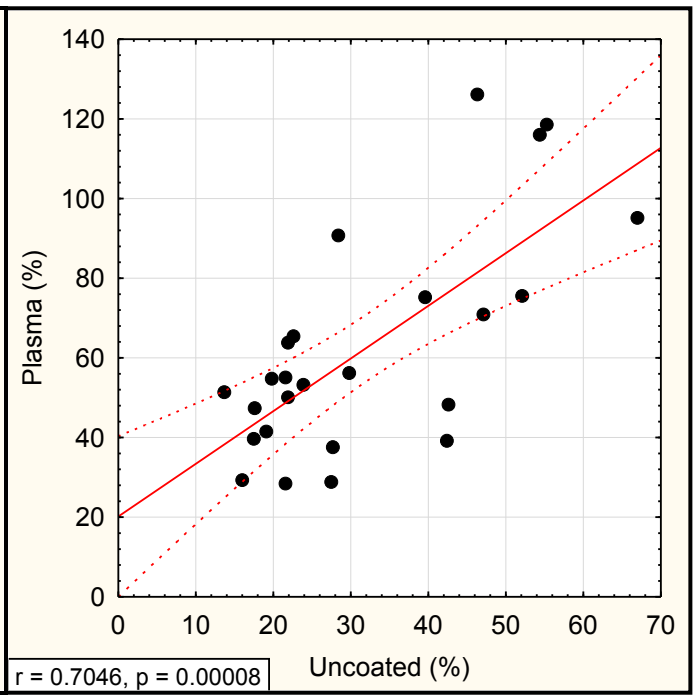


Figure 5.8 Scatter plot of uncoated vs. plasma.

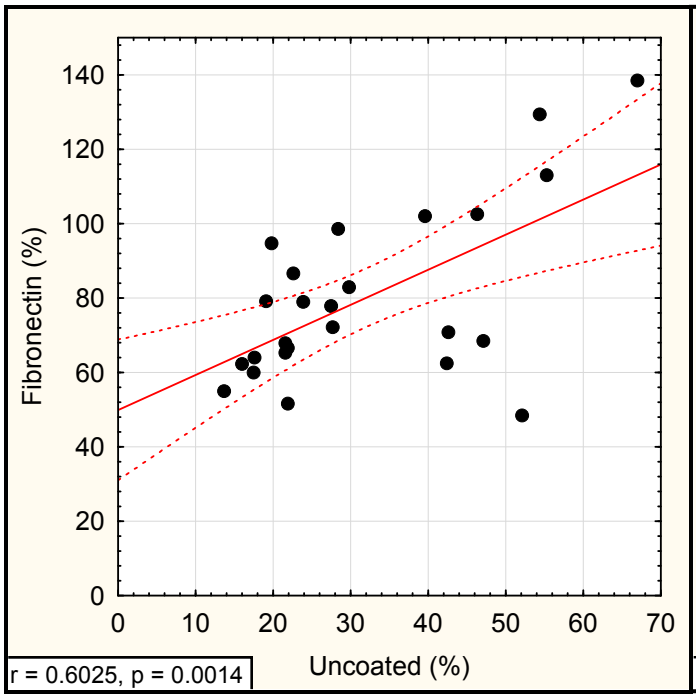


Figure 5.9 Scatter plot of uncoated vs. fibronectin.

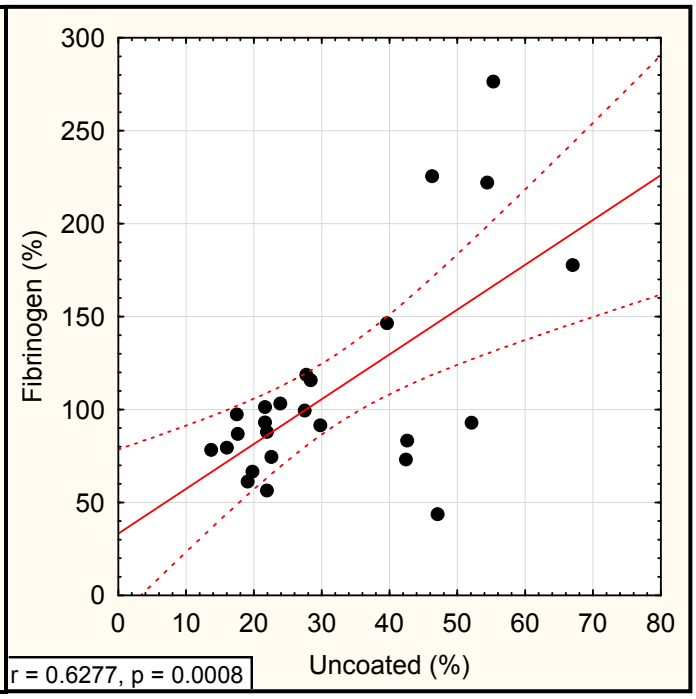


Figure 5.10 Scatter plot of uncoated vs. fibrinogen.

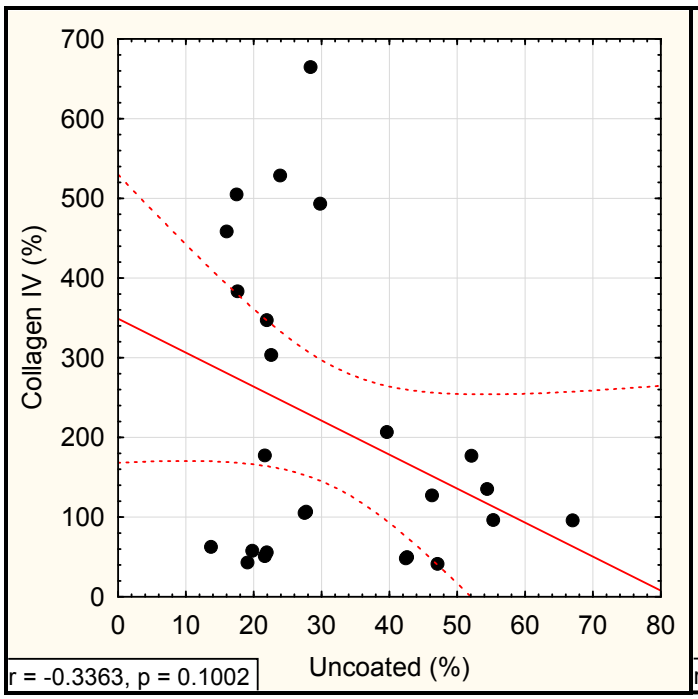


Figure 5.11 Scatter plot of uncoated vs. collagen IV.

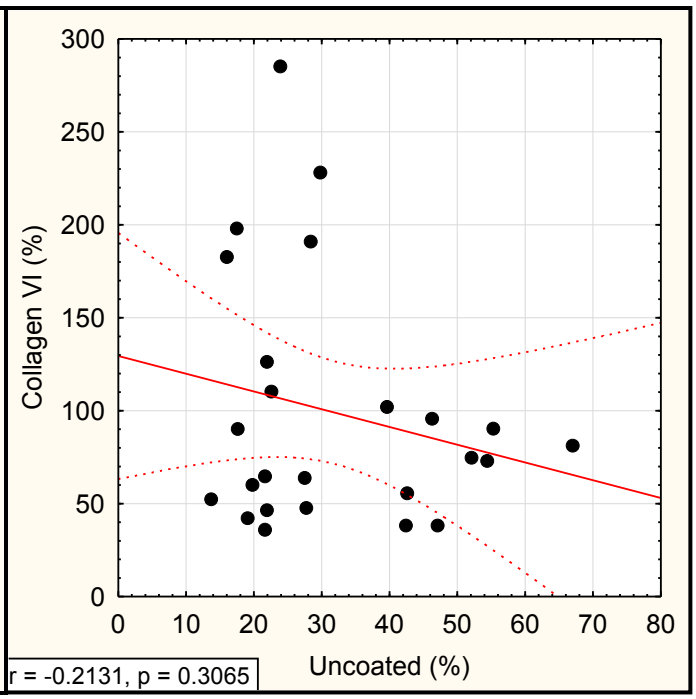


Figure 5.12 Scatter plot of uncoated vs. collagen VI.

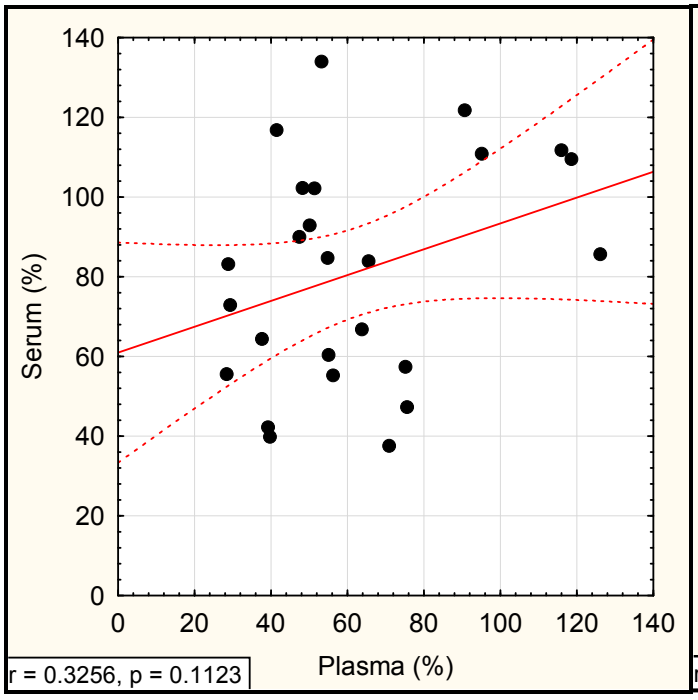


Figure 5.13 Scatter plot of plasma vs. serum.

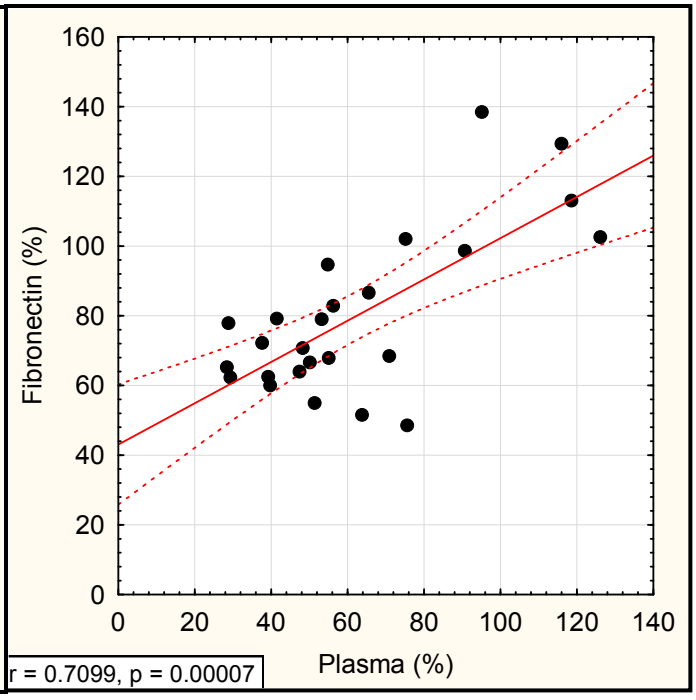


Figure 5.14 Scatter plot of plasma vs. fibronectin.

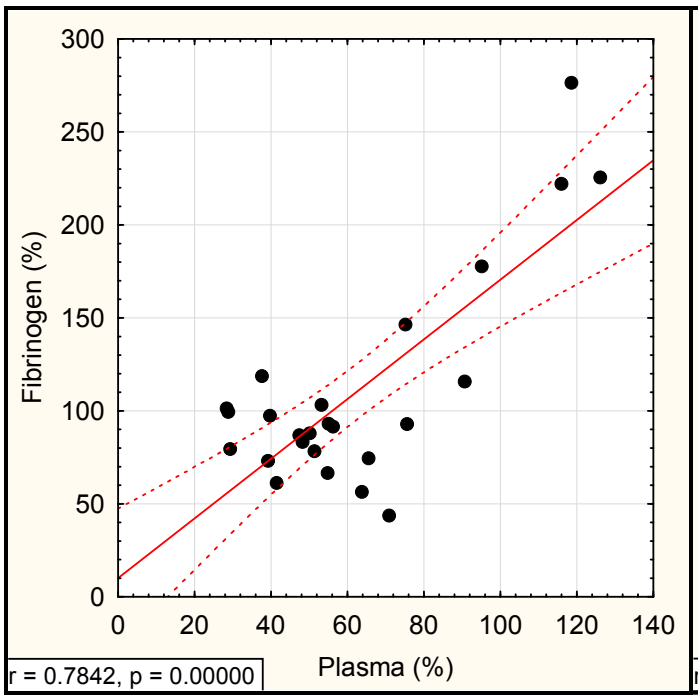


Figure 5.15 Scatter plot of plasma vs. fibrinogen.

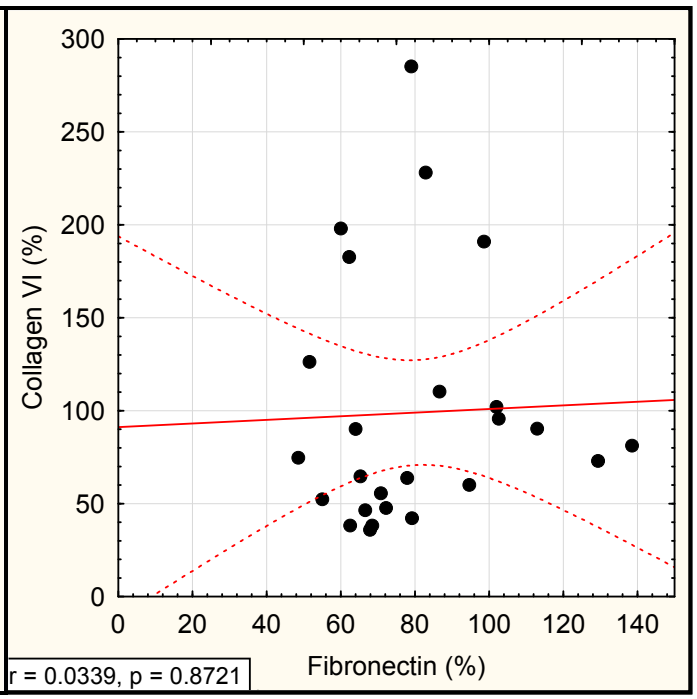


Figure 5.16 Scatter plot of plasma vs. collagen IV.

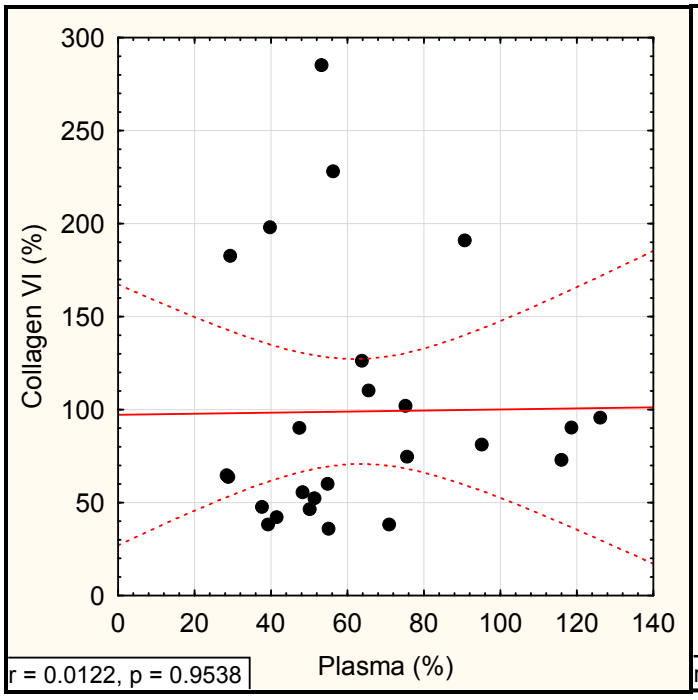


Figure 5.17 Scatter plot of plasma vs. collagen VI.

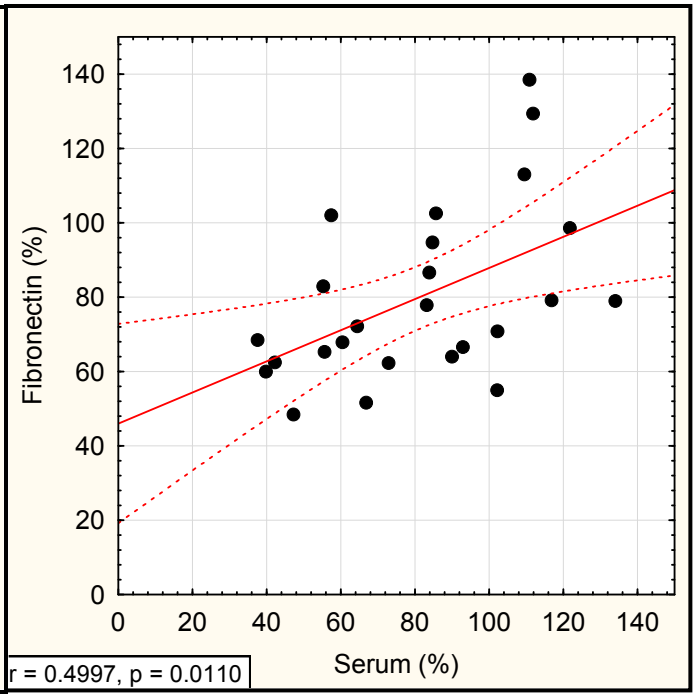


Figure 5.18 Scatter plot of serum vs. fibronectin.

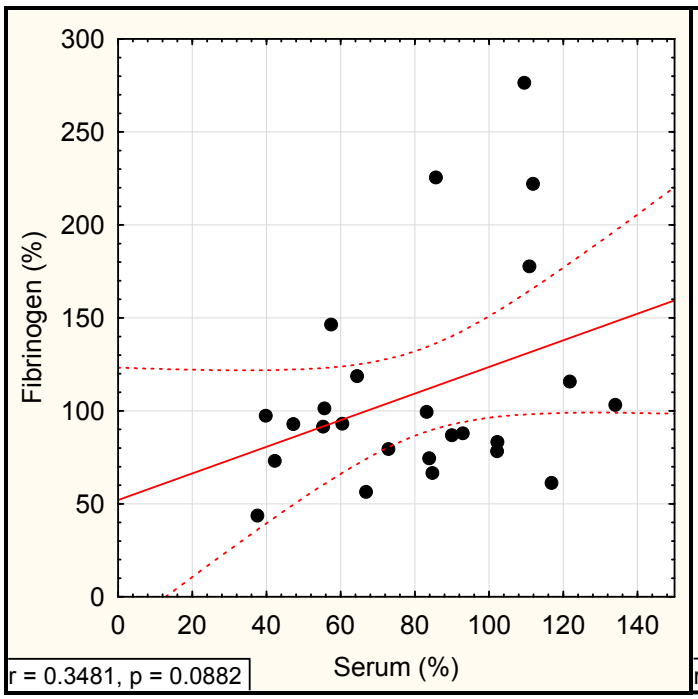


Figure 5.19 Scatter plot of serum vs. fibrinogen.

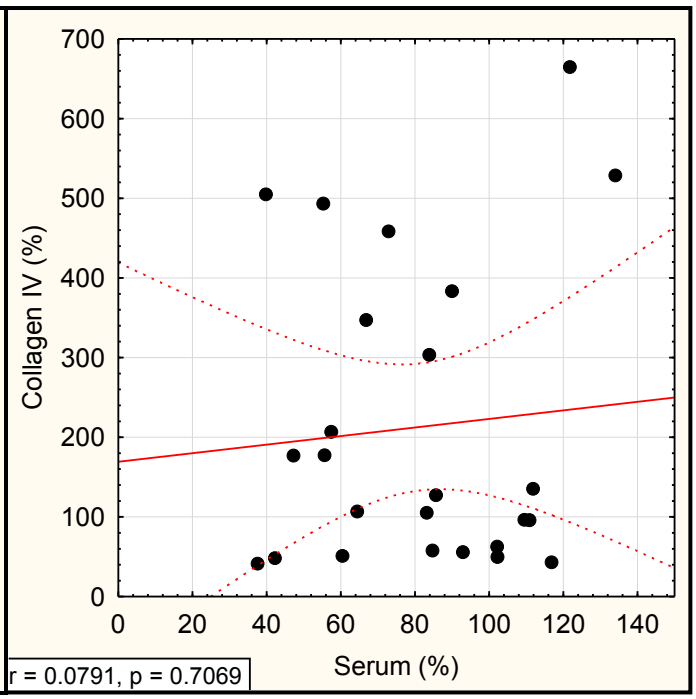


Figure 5.20 Scatter plot of serum vs. collagen VI.

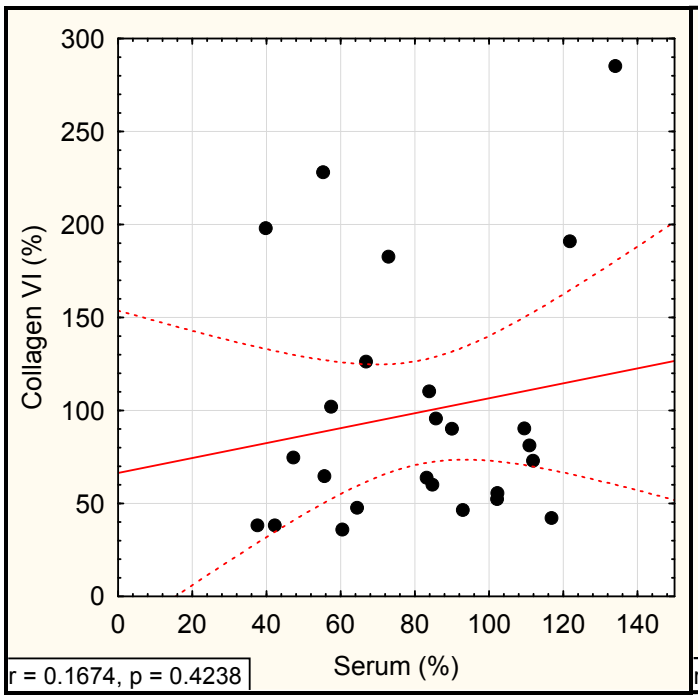


Figure 5.21 Scatter plot of serum vs. collagen VI.

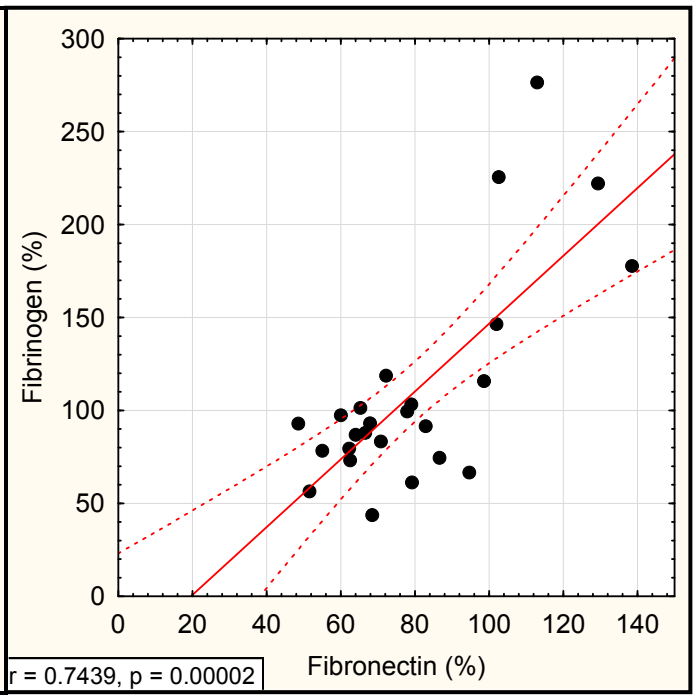


Figure 5.22 Scatter plot of fibronectin vs. fibrinogen.

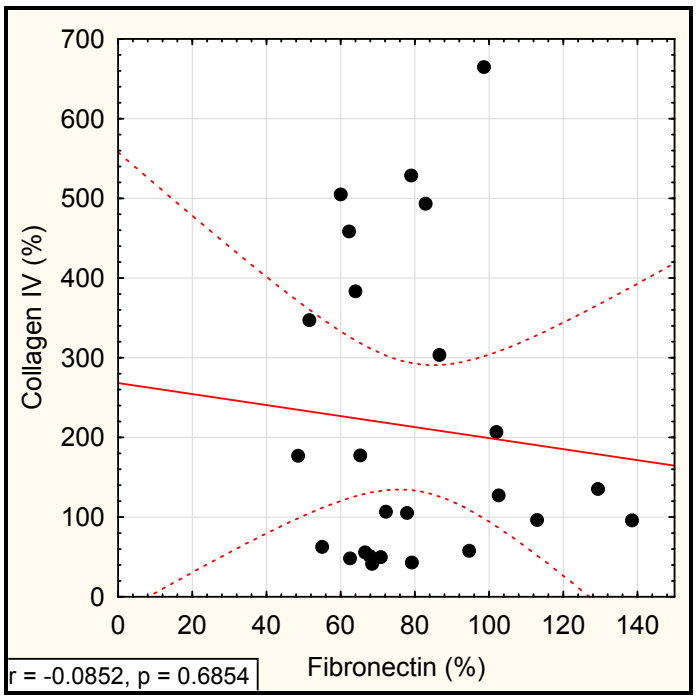


Figure 5.23 Scatter plot of fibronectin vs. collagen VI.

Figure 5.24 Scatter plot of fibronectin vs. collagen VI.

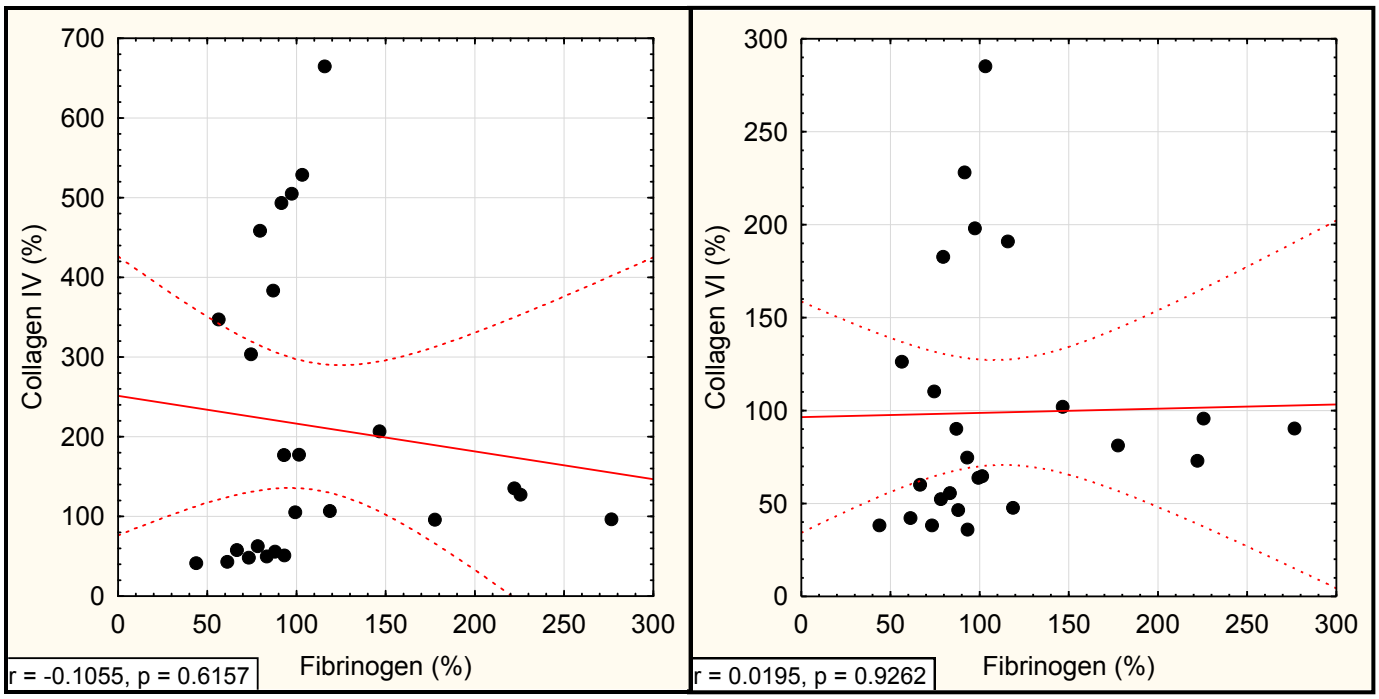


Figure 5.25 Scatter plot of fibrinogen vs. collagen IV.

Figure 5.26 Scatter plot of fibrinogen vs. collagen VI.

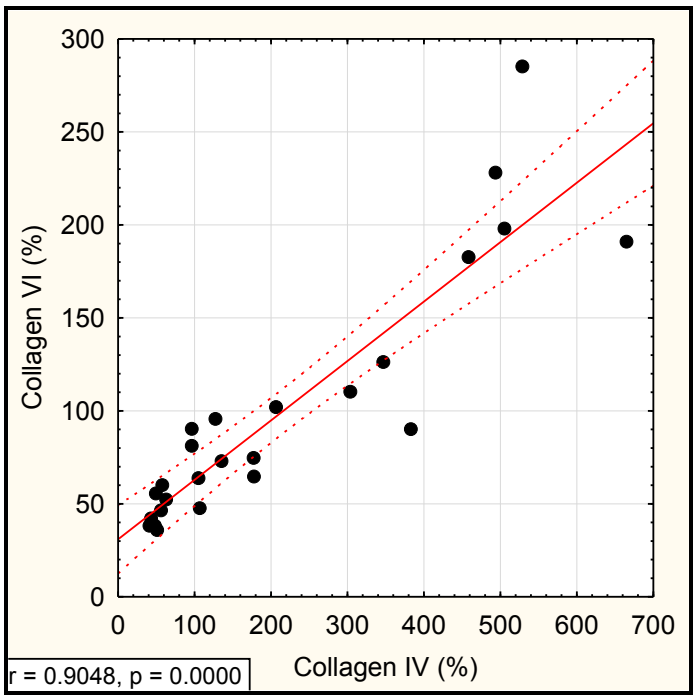


Figure 5.27 Scatter plot of collagen IV vs. collagen VI.

5.2.2.2 Correlations between adherence and invasion assays:

Adherence to serum (Pearson correlation r = 0.5397, p = 0.0065) and fibronectin (r = 0.4702, p = 0.0204) displayed moderate correlation with invasion. The correlation between serum and fibronectin is expected as serum is rich in fibronectin. Since FnBPs are used as the main invasin for *S. aureus*, the ability to bind fibronectin would promote invasion of host cells. Please refer to Table 5.3 for the r and p-values and Figures 5.28 – 5.34 for the scatter plots of the results correlated. A statistical trend was also identified between invasion and adherence to plasma (r = 0.392, p= 0.06). Two subgroups are identified on the scatter plot for collagen IV vs. invasion, of which one displays a linear correlation.

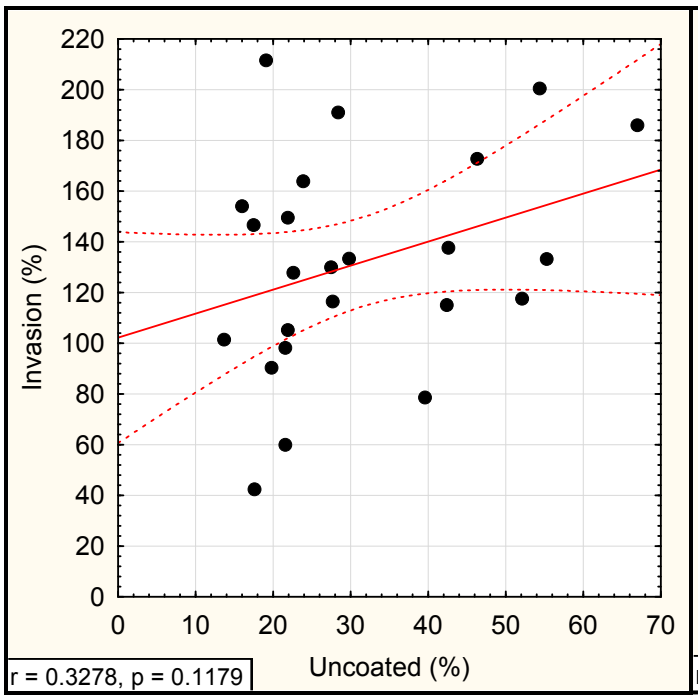


Figure 5.28 Scatter plot of uncoated vs. invasion.

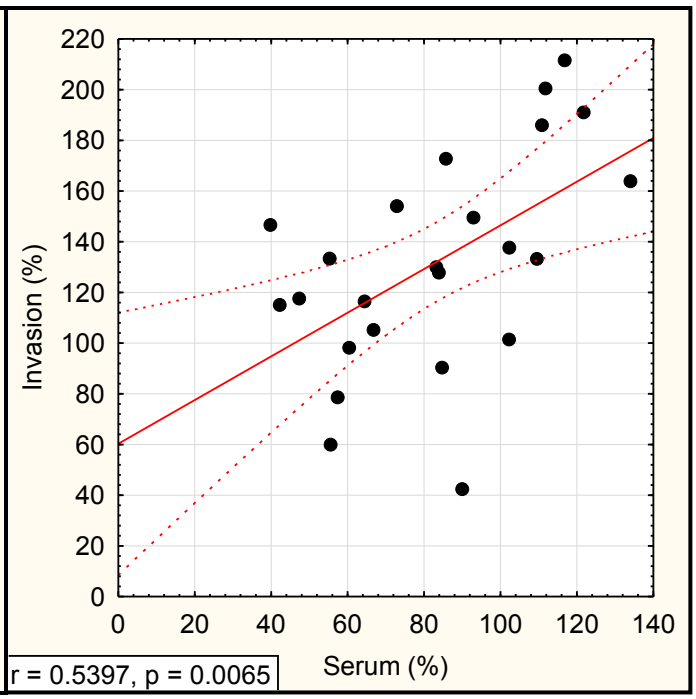


Figure 5.29 Scatter plot of serum vs. invasion.

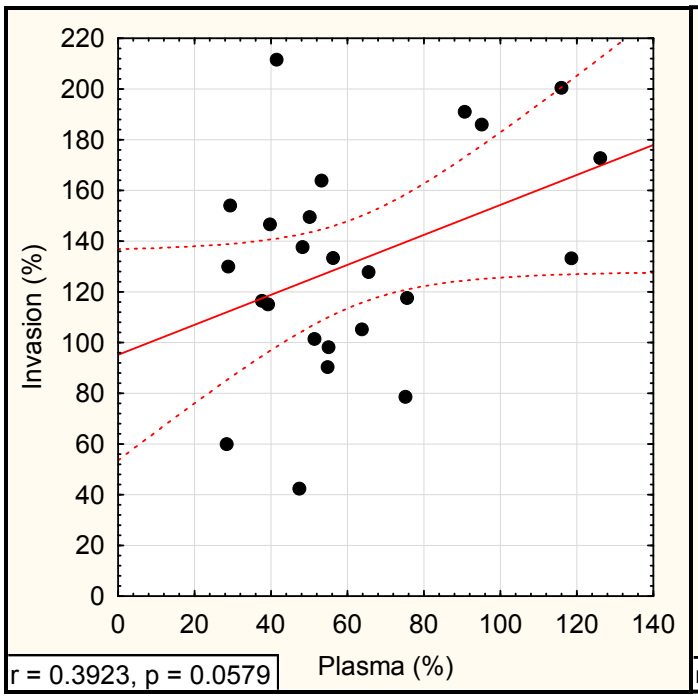


Figure 5.30 Scatter plot of plasma vs. invasion.

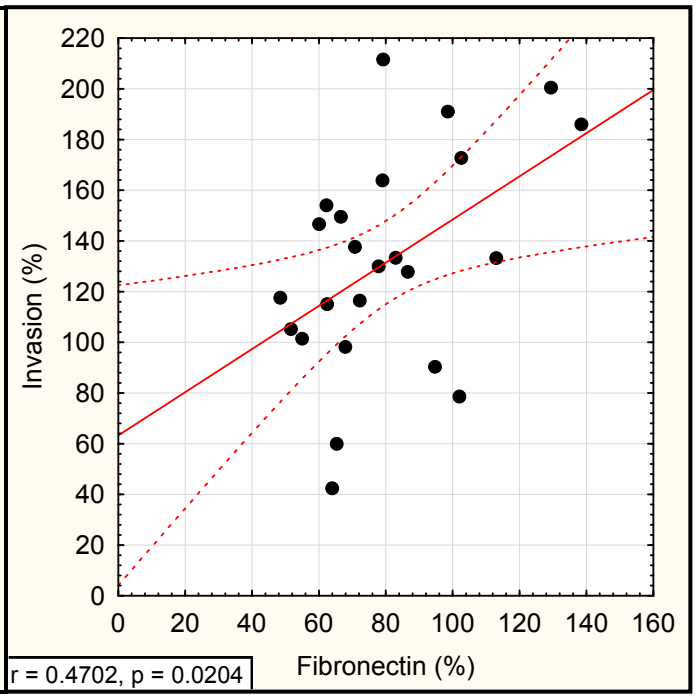


Figure 5.31 Scatter plot of fibronectin vs. invasion.

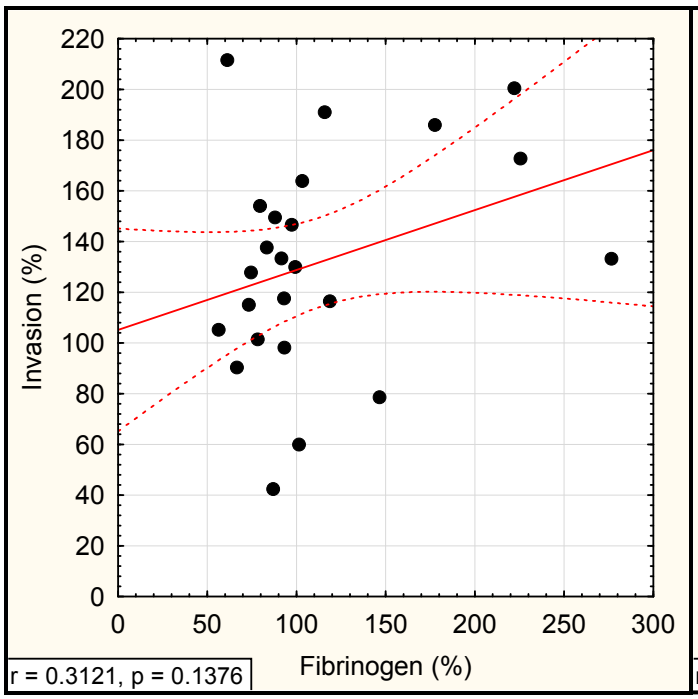


Figure 5.32 Scatter plot of fibrinogen vs. invasion.

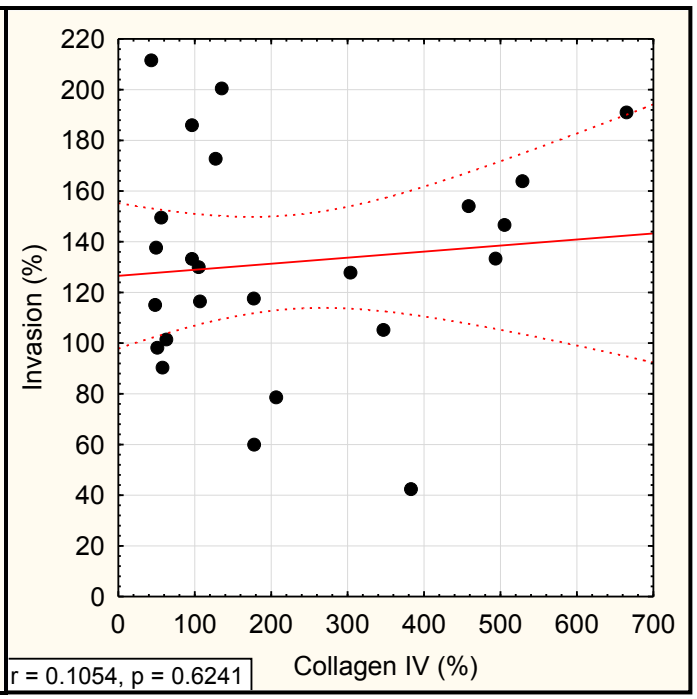


Figure 5.33 Scatter plot of collagen IV vs. invasion.

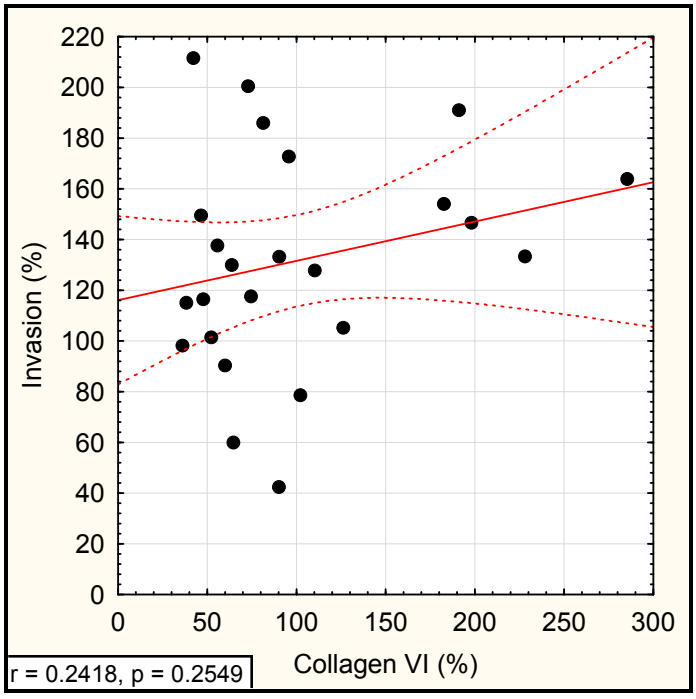


Figure 5.34 Scatter plot of collagen VI vs. invasion.

Table 5.4 Pearson correlation (r) for the correlation between different assays and the corresponding p-value, calculated using the software package STATISTICA v.10. Statistically significant ($p < 0.05$) correlations are displayed in bold.

Assay	Pearson correlation (r)	p-value	Figure
Correlation between adherence and cell death assays			
Serum vs. Nicoletti	0.2947	0.1527	5.35
Uncoated vs. Nicoletti	-0.0577	0.7840	5.36
Plasma vs. Nicoletti	0.0449	0.8313	5.37
Fibronectin vs. Nicoletti	0.2985	0.1473	5.38
Fibrinogen vs. Nicoletti	0.3569	0.0799	5.39
Collagen IV vs. Nicoletti	-0.1097	0.6017	5.40
Collagen VI vs. Nicoletti	0.0741	0.7249	5.41
Serum vs. LDH	0.1975	0.3440	5.42
Uncoated vs. LDH	0.0157	0.9408	5.43
Plasma vs. LDH	0.1604	0.4436	5.44
Fibronectin vs. LDH	0.0799	0.7043	5.45
Fibrinogen vs. LDH	0.3179	0.1215	5.46
Collagen IV vs. LDH	-0.1981	0.3424	5.47
Collagen VI vs. LDH	-0.0911	0.6651	5.48
Serum vs. WST-1	-0.1107	0.5985	5.49
Uncoated vs. WST-1	0.0078	0.9705	5.50
Plasma vs. WST-1	-0.0976	0.6426	5.51
Fibronectin vs. WST-1	-0.2138	0.3049	5.52
Fibrinogen vs. WST-1	-0.3662	0.0718	5.53
Collagen IV vs. WST-1	0.3668	0.0713	5.54
Collagen VI vs. WST-1	0.3006	0.1442	5.55
Correlation between invasion and cell death assays			
Invasion vs. Nicoletti	0.3283	0.1173	5.56
Invasion vs. LDH	0.0768	0.7213	5.57
Invasion vs. WST-1	0.0033	0.9880	5.58
Correlation between difference cell death assays			
Nicoletti vs. LDH	**0.5459**	**0.0048**	**5.59**
Nicoletti vs. WST-1	**-0.7405**	**0.00002**	**5.60**
WST-1 vs. LDH	**-0.6774**	**0.0002**	**5.61**

5.2.2.3 Correlations between adherence and cell death assays:

Adherence to fibrinogen was the only adherence factor which displayed the strongest, yet weak correlations with any of the cell death assays: Nicoletti (Pearson correlation r = 0.3569, p = 0.0799); LDH (r = 0.3179, p = 0.1215); WST-1 (r = -0.3662, p = 0.0718). Adherence to fibronectin displayed even weaker correlations: Nicoletti (r = 0.2985, p = 0.1473); WST-1 (r = -0.2138, p = 0.3049). Adherence to both collagen IV (r = 0.3668, p = 0.0713) and VI (r = 0.3006, p = 0.1442) were weakly correlated only with the WST-1 assay as an indicator of cell death. Please refer to Table 5.4 for the r and p-values and Figures 5.35 – 5.55 for the scatter plots of the results correlated. From these scatter plots we can see that cytotoxic isolates (>50% cytotoxicity) display diversity in their adherence to all of the immobilised ligands investigated. It is also worth mentioning that statistical trends were also identified between adherence to fibrinogen and the Nicoletti assay (r = 0.357. p = 0.08), as well as between adherence to collagen IV and the WST-1 assay (r = 0.367, p = 0.07). This might indicate that adherence to these ligands could be indicators of bacterial cytotoxicity.

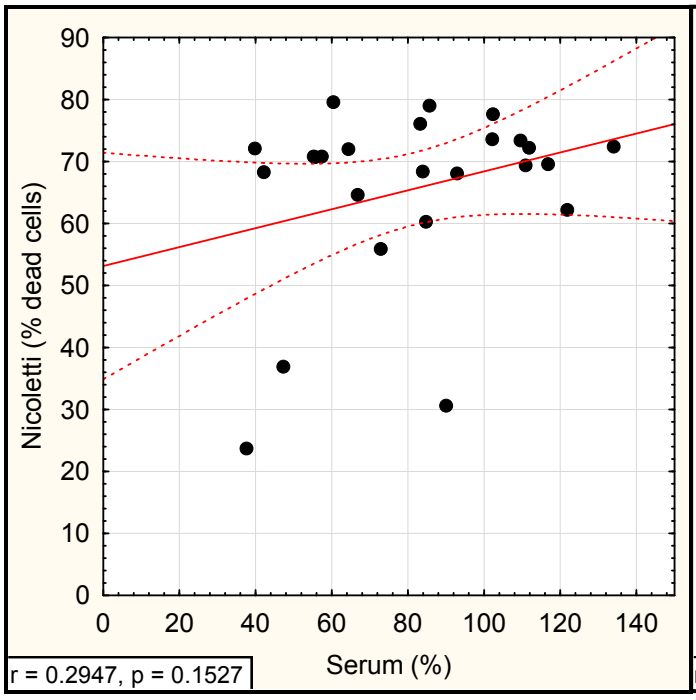


Figure 5.35 Scatter plot of serum vs. Nicoletti.

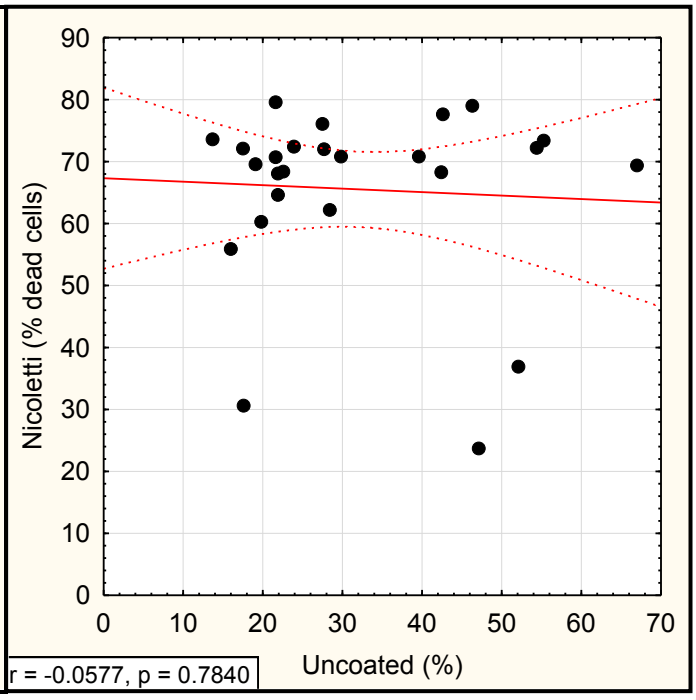


Figure 5.36 Scatter plot of uncoated vs. Nicoletti.

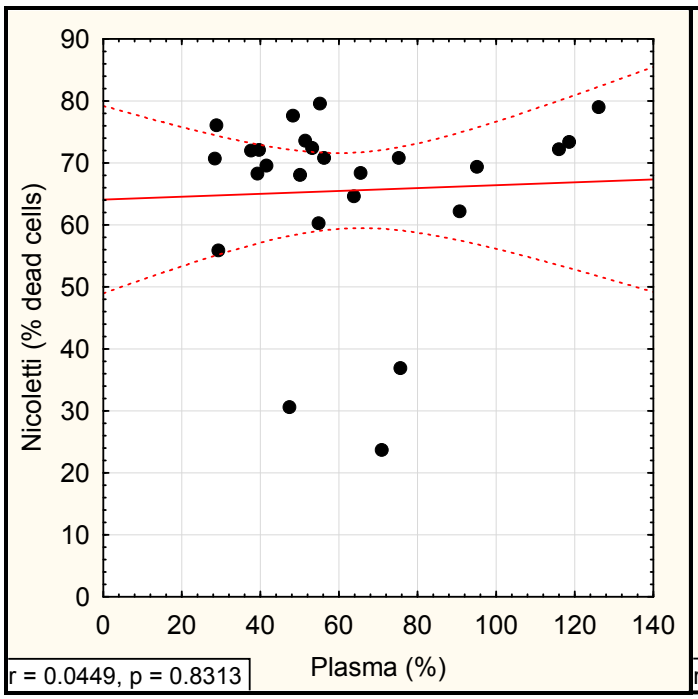


Figure 5.37 Scatter plot of plasma vs. Nicoletti.

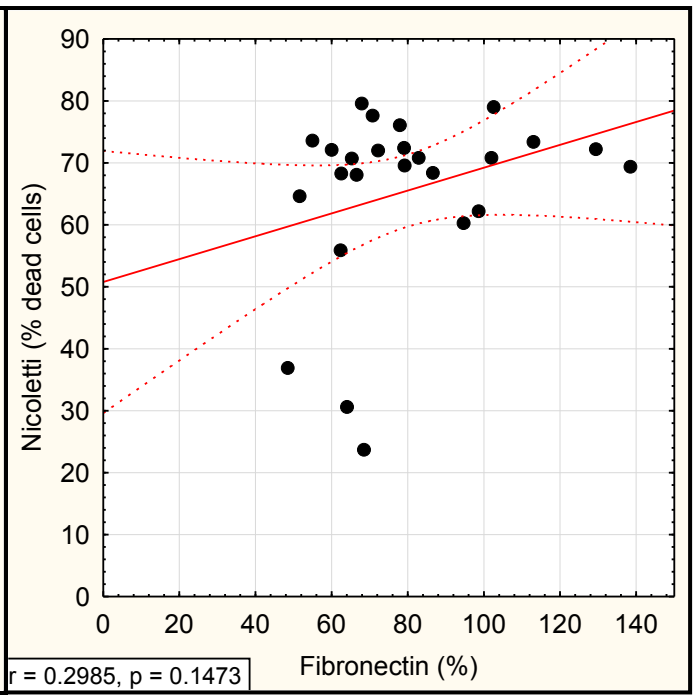


Figure 5.38 Scatter plot of fibronectin vs. Nicoletti.

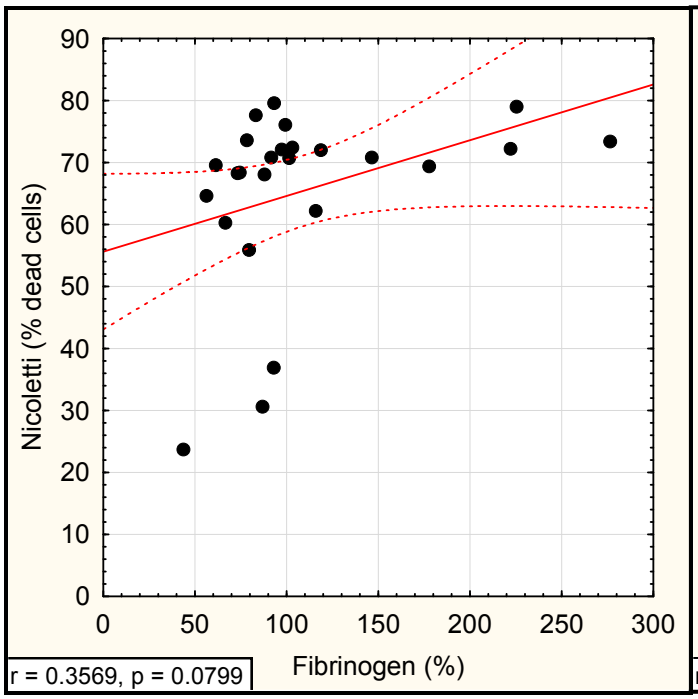


Figure 5.39 Scatter plot of fibrinogen vs. Nicoletti.

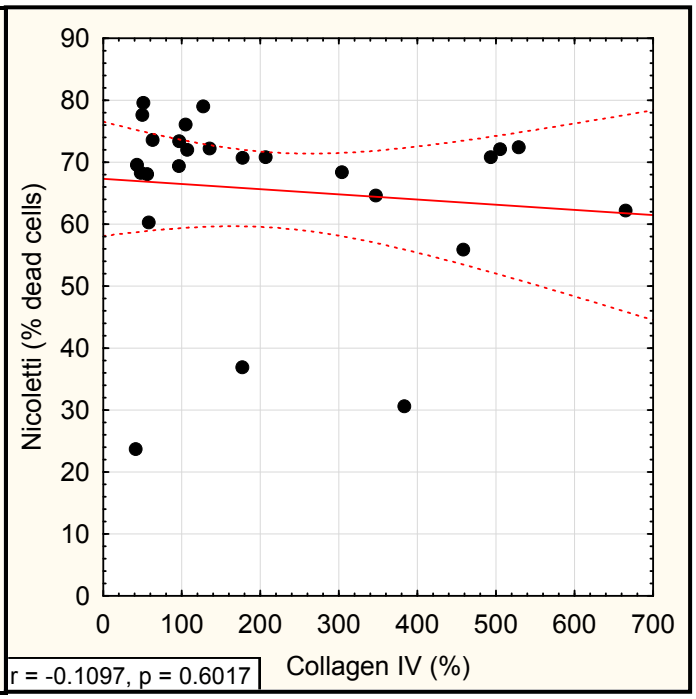
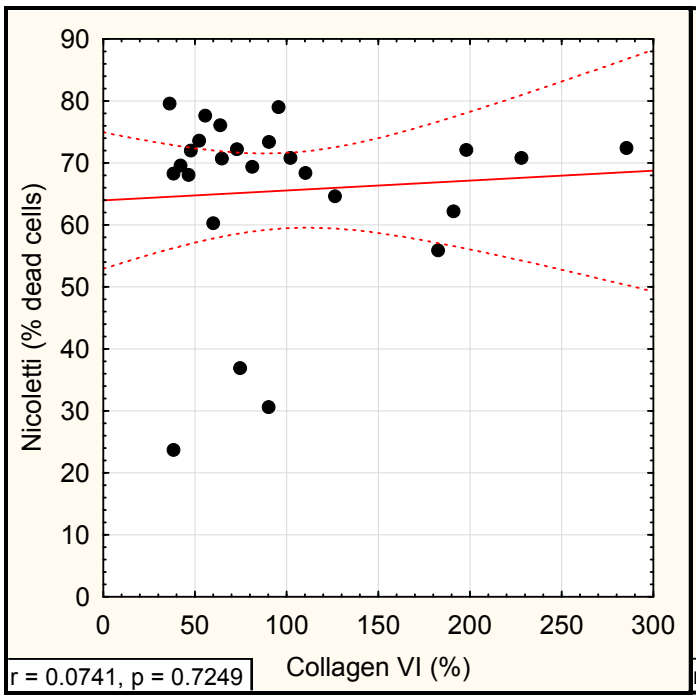


Figure 5.40 Scatter plot of collagen IV vs. Nicoletti.



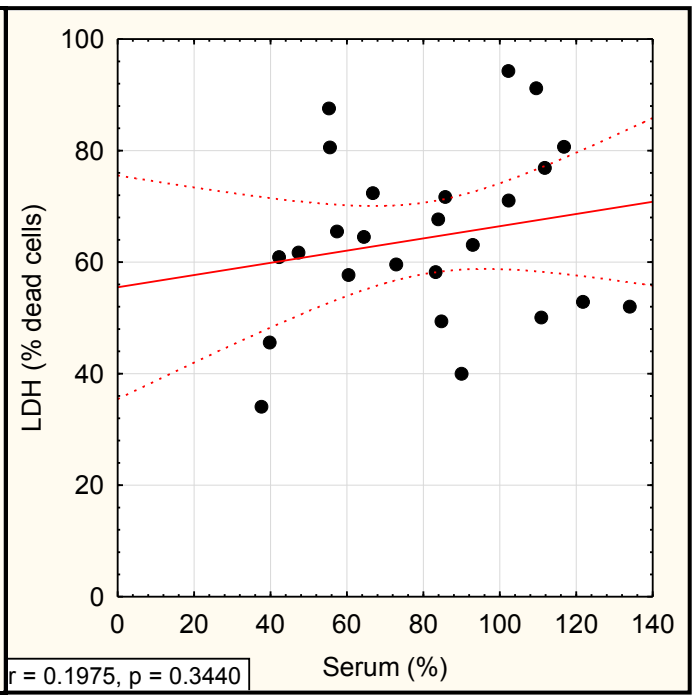
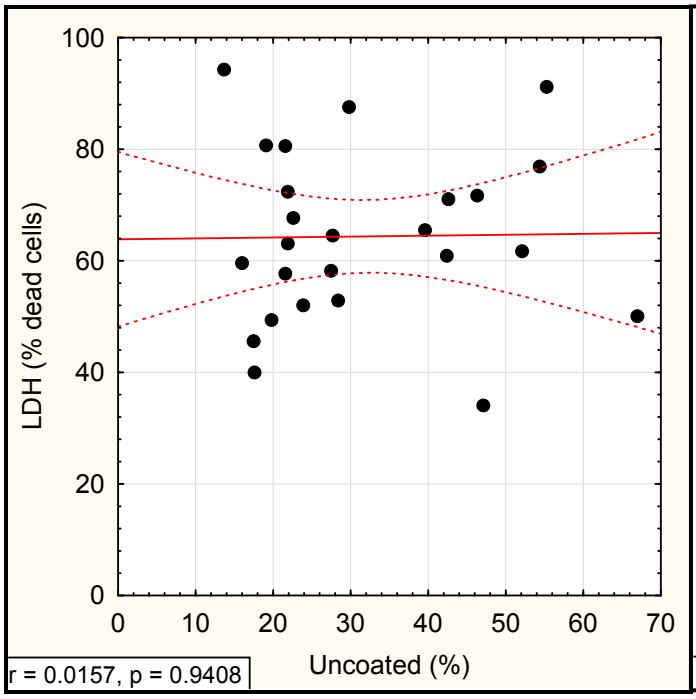


Figure 5.41 Scatter plot of collagen VI vs. Nicoletti. **Figure 5.42** Scatter plot of serum vs. LDH.



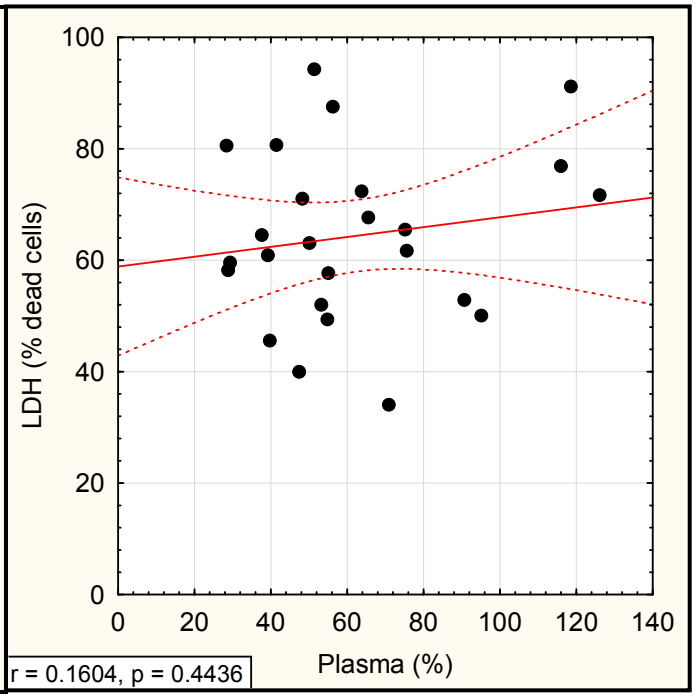


Figure 5.43 Scatter plot of uncoated vs. LDH. **Figure 5.44** Scatter plot of plasma vs. LDH.

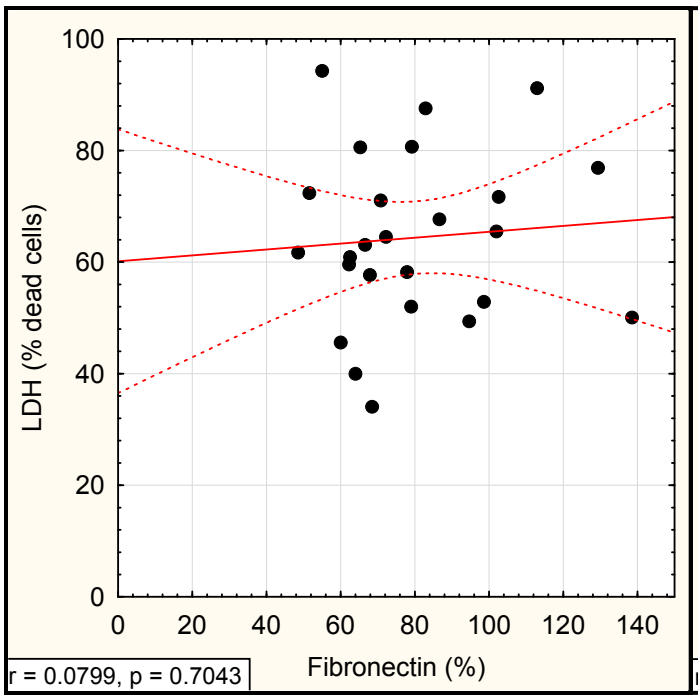


Figure 5.45 Scatter plot of fibronectin vs. LDH.

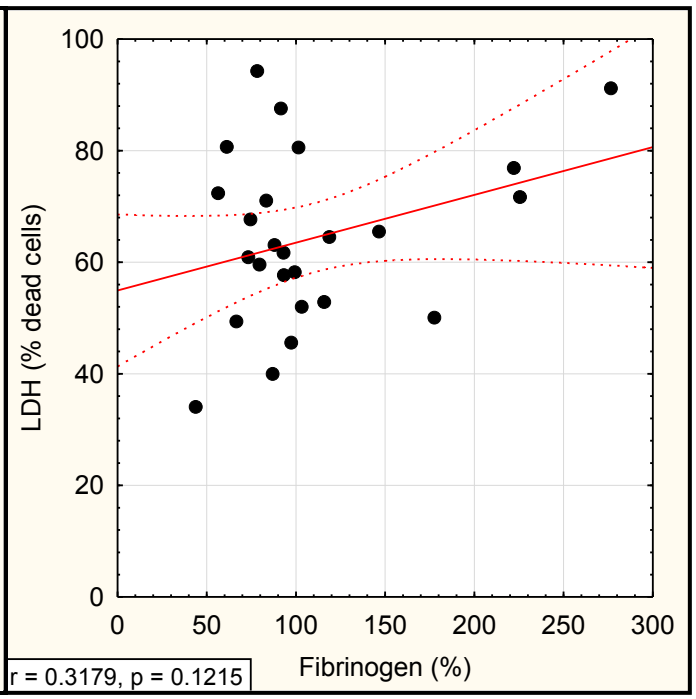


Figure 5.46 Scatter plot of fibrinogen vs. LDH.

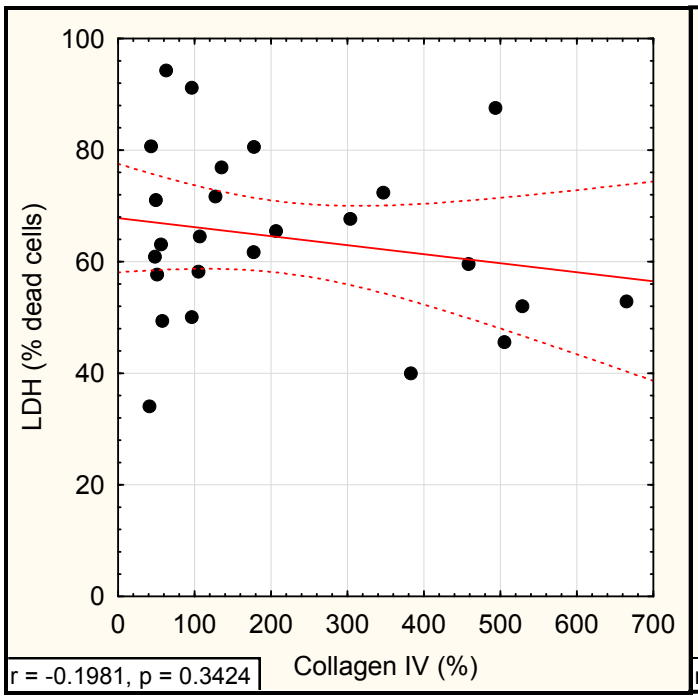


Figure 5.47 Scatter plot of collagen IV vs. LDH.

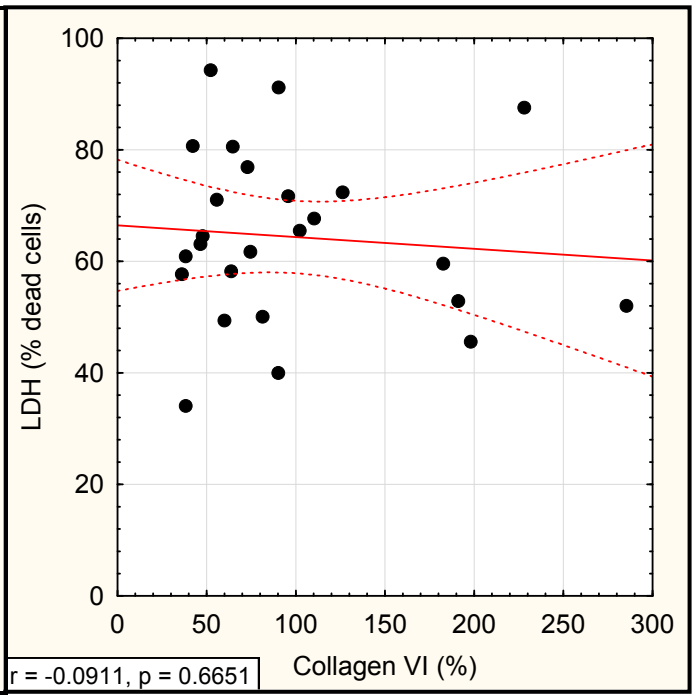


Figure 5.48 Scatter plot of collagen VI vs. LDH.

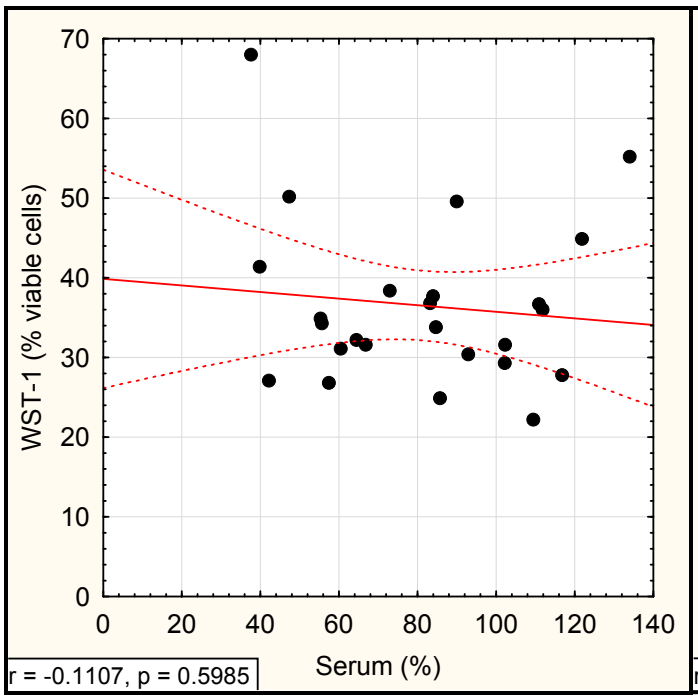


Figure 5.49 Scatter plot of serum vs. WST-1.

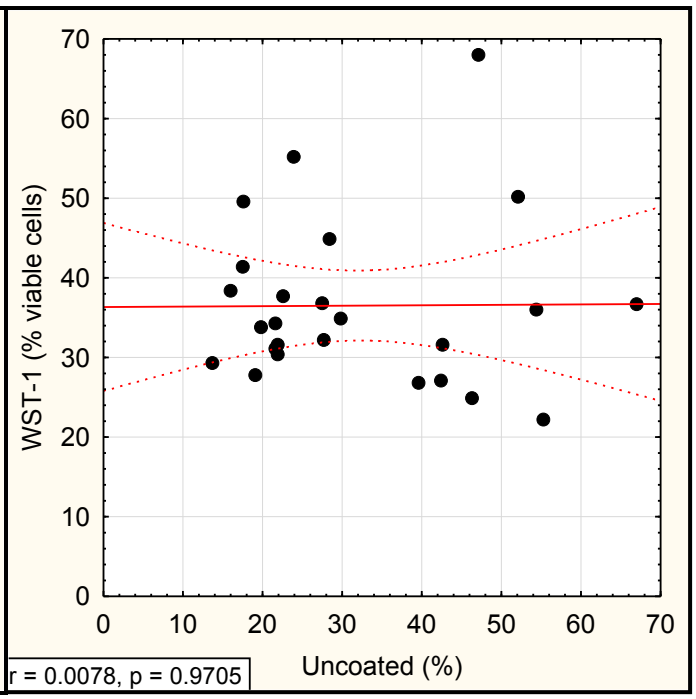


Figure 5.50 Scatter plot of uncoated vs. WST-1.

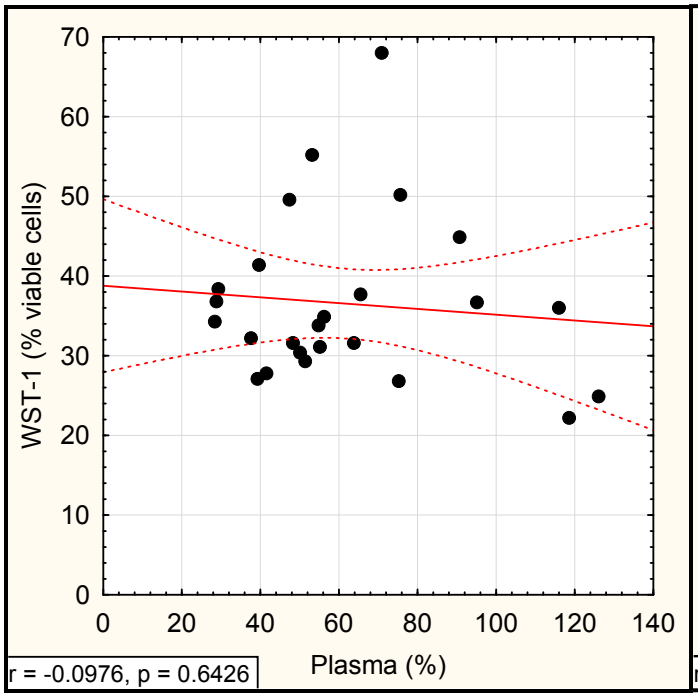


Figure 5.51 Scatter plot of plasma vs. WST-1.

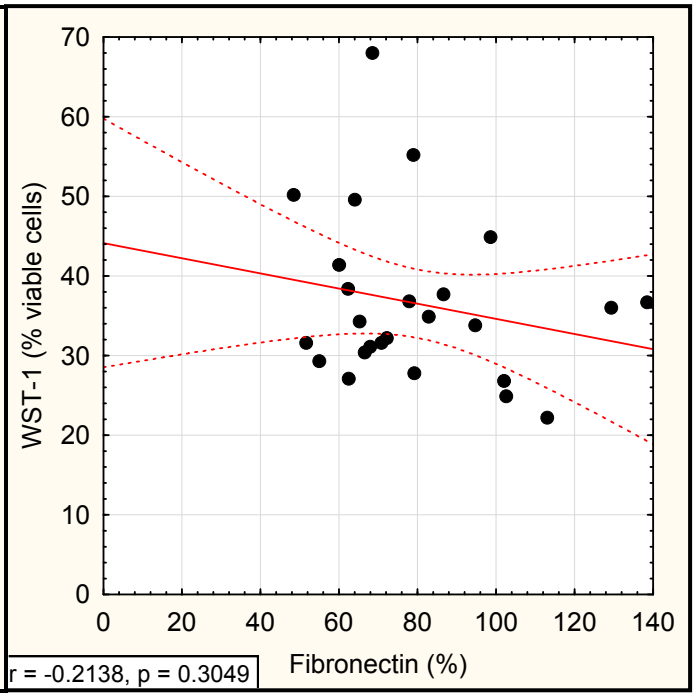


Figure 5.52 Scatter plot of fibronectin vs. WST-1.

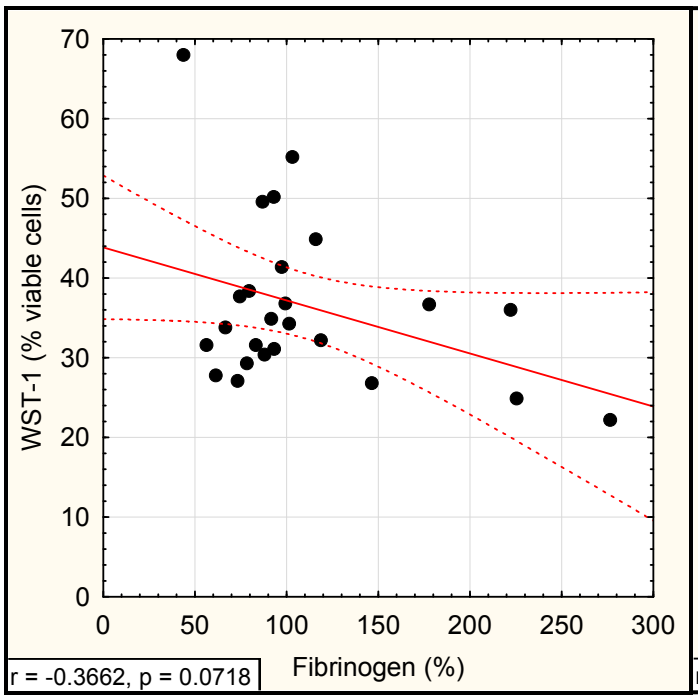


Figure 5.53 Scatter plot of fibrinogen vs. WST-1.

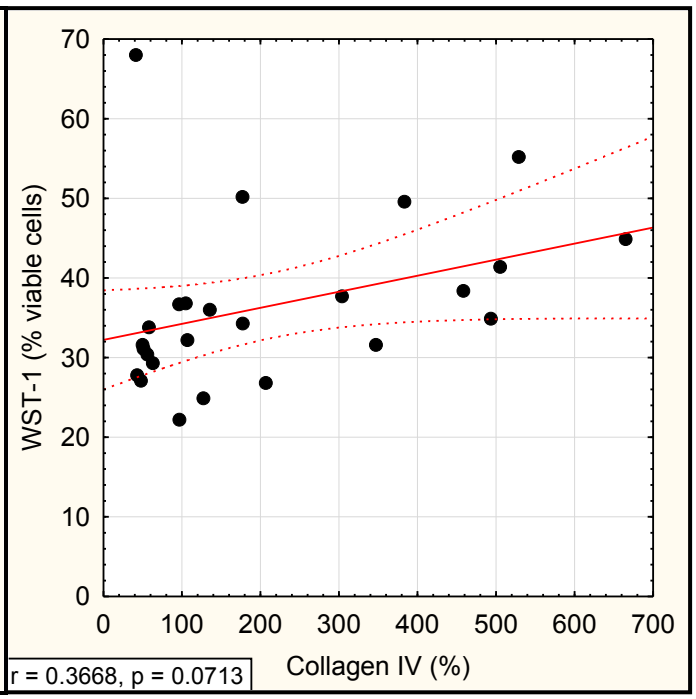


Figure 5.54 Scatter plot of collagen IV vs. WST-1.

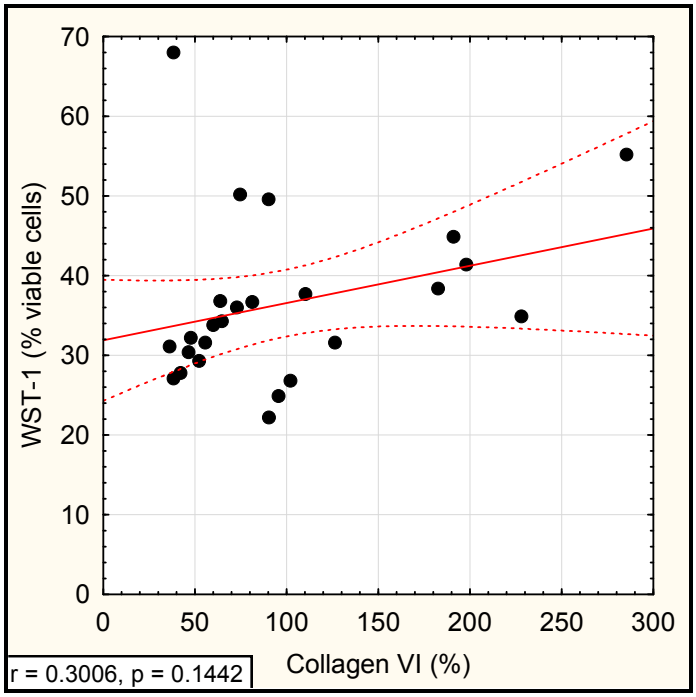
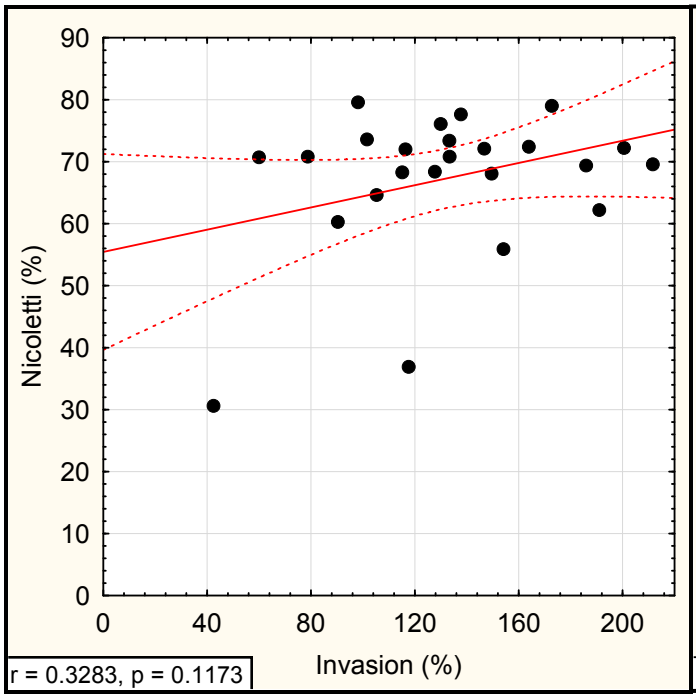


Figure 5.55 Scatter plot of collagen VI vs. WST-1.

5.2.2.4 Correlations between invasion and cell death assays:

The ability to invade host cells only correlated weakly with the Nicoletti assay as an indicator of cell death (Pearson correlation r = 0.3283, p = 0.1732). From this we can gather that cytotoxic isolates are invasive and that invasiveness is required for cytotoxicity. Please refer to Table 5.4 for the r and p-values and Figures 5.56 – 5.58 for the scatter plots of the results correlated.



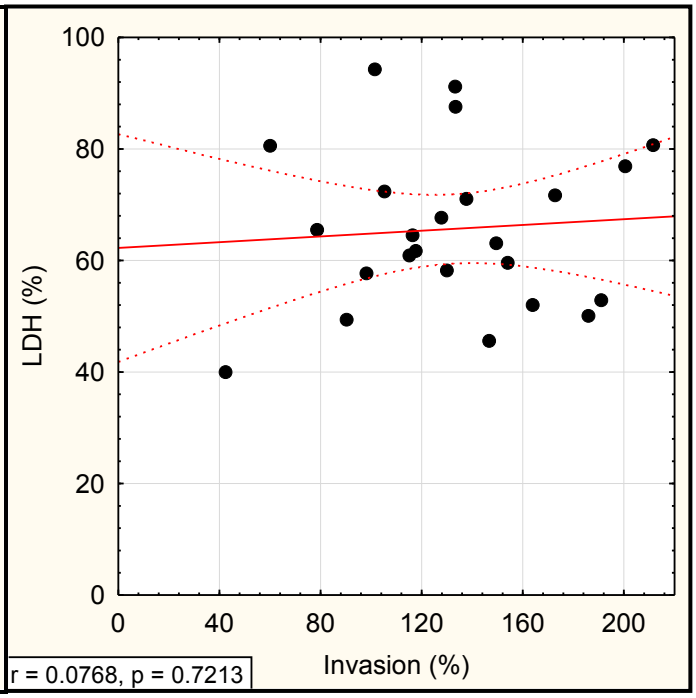


Figure 5.56 Scatter plot of invasion vs. Nicoletti. **Figure 5.57** Scatter plot of invasion vs. LDH.

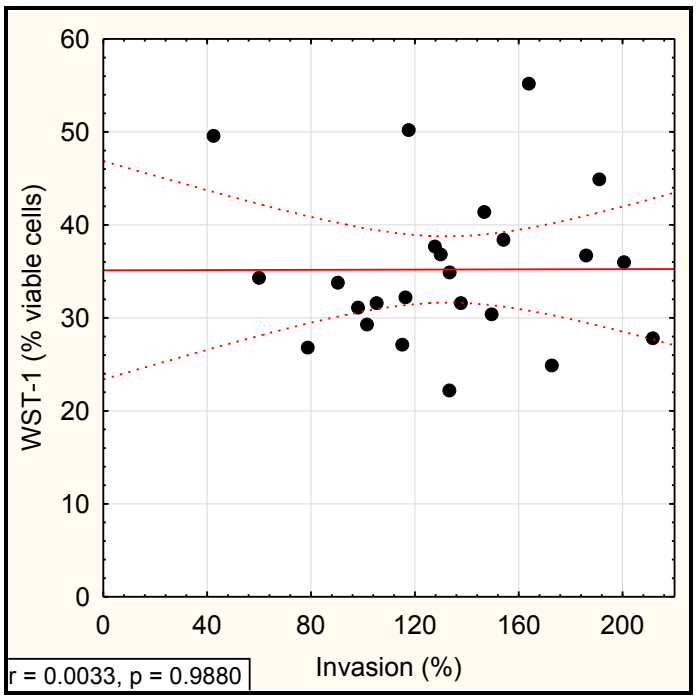


Figure 5.58 Scatter plot of invasion vs. WST-1.

5.2.2.5 Correlations between cell death assays:

Strong correlations were identified between the 3 different *in vitro* assays used as indicators of cell death. The strongest correlation was identified between the Nicoletti and WST-1 assays (Pearson correlation r = -0.7405, p = 0.00002), followed by the WST-1 and LDH assays (r = -0.6774, p = 0.0002), and the weakest correlation was between the Nicoletti and LDH assays (r = 0.5459, p = 0.0048). The lack of correlation between the Nicoletti and LDH assays might be due to the different end points measured (Nicoletti measures hypodiploid nuclei; LDH measures release of LDH from cell), which might indicate different mechanisms of cell death induction. Some isolates might favour a single mechanism if inducing cell death, whereas other isolates might be capable of inducing host cell death using a variety of mechanisms. Please refer to Table 5.4 for the r and p-values and Figures 5.59 – 5.61 for the scatter plots of the results correlated.

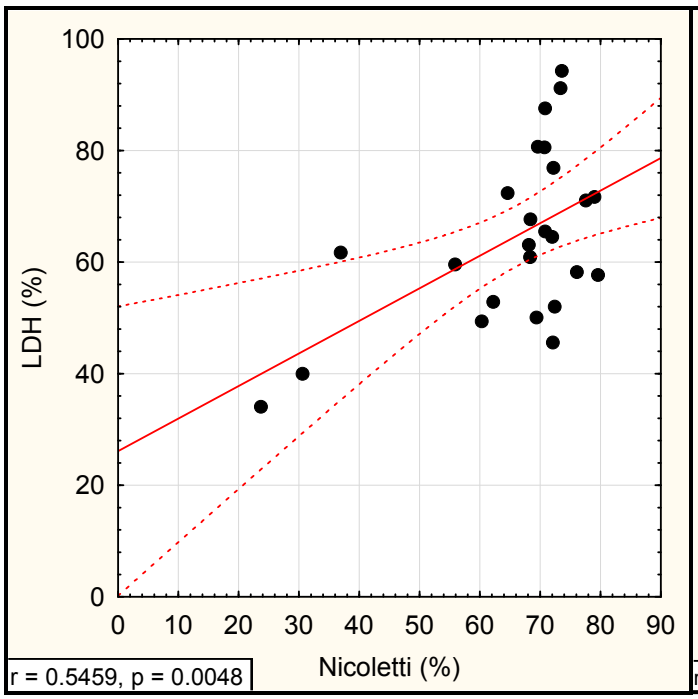


Figure 5.59 Scatter plot of Nicoletti vs. LDH.

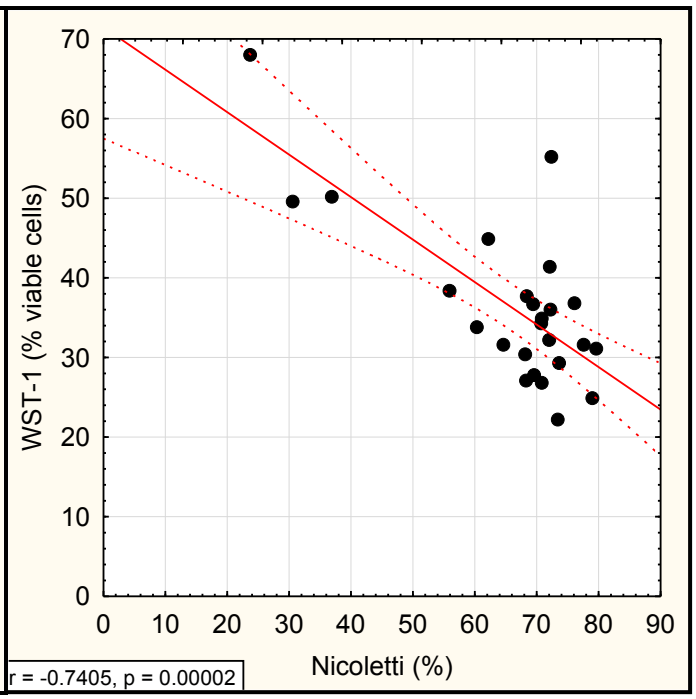


Figure 5.60 Scatter plot of Nicoletti vs. WST-1.

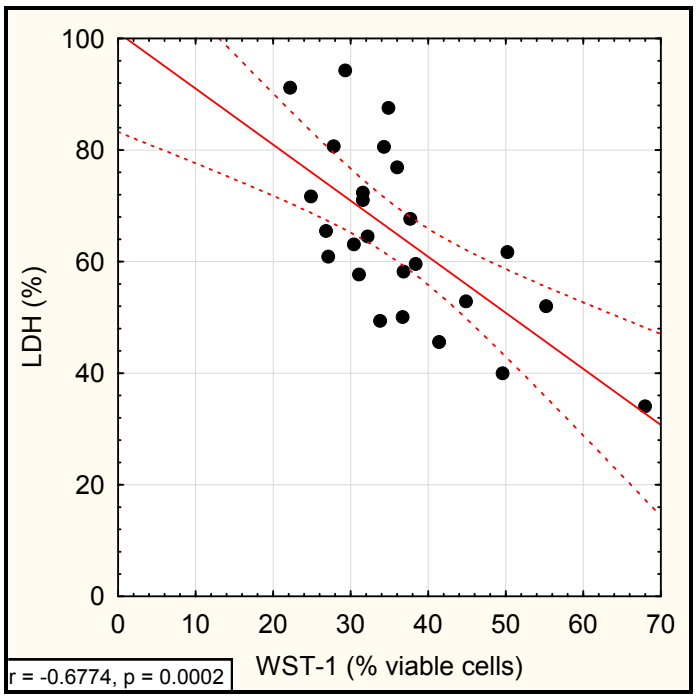


Figure 5.61 Scatter plot of WST-1 vs. LDH.

CHAPTER 6: General conclusions

Data on the population structure of *S. aureus* isolates from specific clinical sites at Tygerberg hospital was lacking. Furthermore, no study has previously been conducted to investigate the differences in virulence of various clones in circulation in South Africa. This study was designed to identify important clones circulating in the patient population service by Tygerberg hospital and then to investigate the differences in *in vitro* virulence of these clones using surrogate parameters of virulence.

From this investigation we concluded that this population of *S. aureus* consist of a diverse number of endemic MSSA and epidemic MRSA clones and that we attempted to associate with various clinical or bacterial characteristics, including source of infection, gender and HIV status. As we were able to demonstrate new SCC*mec* acquisitions in the population of MSSA that we investigated, we can speculate that, upon acquisition of SCC*mec* elements by this population harbouring a remarkably high incidence of PVL encoding genes, highly virulent strains may emerge which can possibly lead to outbreaks similar to the CA-MRSA epidemics described in the literature elsewhere.

ST612-MRSA-IV was identified as the dominant MRSA clones, previously only described in South Africa and Australia.

Isolates selected as representatives possessed a diverse pattern of virulence genes determined by PCR, of which some were associated with certain clones or with methicillin-resistance or susceptibility. Some genes, such as *fnbA/B, clfA/B, eap* and *hld* were identified in all the representative isolates. These representative isolates also displayed diverse preferences for various immobilised human ligands. The vast majority of representative isolates were both highly invasive and cytotoxic. We can thus conclude that this *S. aureus* population consist out of a variety of PVL-MRSA and both PVL- and PVL+ MSSA clones, of which representatives displayed diversity in adherence to monomeric immobilised human ligands and are both highly invasive and cytotoxic.

The population structure data generated also identified the following prominent findings: (1) a lack of CA-MRSA clones; (2) a lack of PVL+ MRSA clones; (3) a lack of a common genetic

background between MRSA and MSSA clones; (4) a significantly higher PVL prevalence when compared to previously published data, especially among MSSA clones; (5) the local emergence of three MRSA clones; and (6) high prevalence of genes associated with cellular adherence and invasion, induction of host cell death and host immune evasion.

The *in vitro* host-pathogen interaction assays identified the following prominent findings: (1) MRSA isolates adhered stronger to plasma and in the absence of a ligand than MSSA; (2) our isolates displayed diversity in adherence to various immobilised ligands, where for some adherence was a strain-specific, and for others a clonal characteristic; (3) neither invasiveness nor cell death induction was associated with methicillin-resistance; (4) the vast majority of representative isolates were invasive; (5) the vast majority of representative isolates were cytotoxic; (6) no significant difference in adherence, invasiveness or cell death induction was observed between isolates from HIV+ and HIV- persons; and (7) PVL- isolates were more invasive than their PVL+ counterparts.

To our knowledge, this is the first study investigating the differences in *in vitro* functional parameters of virulence (i.e. adherence, cellular invasion and cytotoxicity) of various *S. aureus* clones from the African continent. This is also the first comprehensive study to investigate the population structure of *S. aureus*, isolated from specific clinical sources, at Tygerberg hospital. No data concerning the *agr* types and prevalence of virulence genes from South African *S. aureus* isolates are currently available. Of the numerous MRSA clones studied, ST612-MRSA-IV is unique as it has only been identified in South Africa and Australia, and seems to be a dominant MRSA clone in South Africa. The dominant MSSA clone, ST22-MSSA could acquire methicillin-resistance and lead to CA-MRSA infections and possibly outbreaks as a single ST22-MRSA-V isolate was also identified, together with four ST22-MRSA-IV isolates.

Strengths of the study include: (1) elucidating the molecular epidemiology of the collection of isolates using the gold standard technique, supplemented with other well established techniques (comprehensive investigation); (2) associating prevalent clones with specific clinical/bacterial characteristics; (3) identifying novel *spa* types and MLST sequence types; (4) identifying clones associated with HIV+ status; (5) providing the first basic data on *in vitro* host-pathogen interaction from South African *S. aureus* isolates; and (6) no blood culture isolates were included, as blood culture isolates may originate from any specific site of infection.

Limitations of the study include: (1) severe hampering of the identification of more clones associated with HIV+ status due to the unknown HIV status in the majority of patients; (2) only isolates from specific clinical sources were included, not blood culture isolates; (3) host-pathogen

aspects investigated were relatively non-specific, broad and included only a single isolate for many PFGE clones and MLST STs; (4) no molecular mechanisms were investigated; and (5) no *in vivo* data was obtained.

Several aspects of interest could not be covered by this study. It would be of interest to study the presence of any of the identified clonal complexes in livestock from the same geographical area. This could be coupled with molecular risk assesments to investigate the spreading of virulent clonal complexes. Patient outcome was also not investigated. The molecular epidemiology and host-pathogen investigation performed during this study could be coupled with patient outcome investigation for a more detailed clinical–epidemiolical study.

Several new questions have arisen and this research can lead to the initiation of new projects by investigating, for example, the mechanism(s) used by these isolates to induce cell death. Also, the gene expression profiles, focussing on selected virulence genes, could be analysed to investigate and establish exactly which genes are involved in the infection process during cellular invasion and host cell death induction. Knock-out mutants could also be created and their functional behaviour compared to those of the parental wild-type isolate to investigate molecular mechanisms. Also, more isolates can be selected for analyses, especially from the clones for which only one isolate was originally included. *In vivo* infection models are currently available which could be utilised to investigate the *in vivo* virulence of isolates. More detailed retrospective clinical information obtained from patient files could shed light on the clinical course of infection, including antibiotic treatment, as well as patient outcome.

CHAPTER 7: References

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